



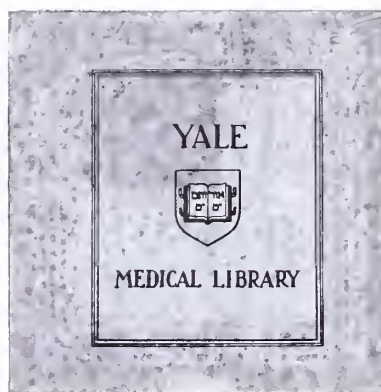
CIRCULATING PROLACTIN LEVELS IN  
POTASSIUM LOADED RATS



Christopher Newport Otis

1982









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CIRCULATING PROLACTIN LEVELS IN  
POTASSIUM LOADED RATS

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A Thesis Submitted to the Yale University School of Medicine  
in Partial Fulfillment of the Requirements for the Degree of  
Doctor of Medicine, 1982.





For Gail, who often took concerns of mine and made them her own.



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## ABSTRACT

Circulating prolactin levels in adolescent male rats were determined by radioimmunoassay in three separate studies to determine if a relationship between chronic oral potassium load and circulating prolactin levels exists. All studies employed adolescent Sprague-Dawley rats fed approximately ten times the amount of potassium contained in the control group diet. Tap water was offered ad lib. Care was taken to minimize acute stress stimuli. All animals were sacrificed by rapid decapitation. Animals were sacrificed at 1500 hr. in the pilot and longitudinal studies. In the third study, animals were sacrificed at 1800 hr.

Plasma prolactin levels were approximately 78% lower in experimental rats after three weeks of potassium adaptation compared to control rats in the pilot study and longitudinal study. There was a small but nonsignificant rise in plasma prolactin after seven days of oral potassium load compared to the control group in the longitudinal study. Linear regression analysis of the points described by control and experimental group mean plasma prolactin levels versus time showed no significant difference in the slopes of experimental and control lines. There was no significant difference in serum prolactin levels in the third study after five days of oral potassium load compared to controls.

Serum osmolality was determined by freezing point depression in the third study. A significant rise ( $P < 0.01$ ) in serum osmolality was noted after five days of potassium load ( $292 \pm 1.80$  mosm/kg) compared to the control group at the start of oral potassium adaptation ( $284 \pm$



0.91 mosm/kg, mean  $\pm$  SEM). There was no difference between serum osmolality in the control group and experimental group after five days of potassium adaptation. Previous investigations have shown a positive correlation between serum osmolality and circulating prolactin levels.

Although serum osmolality was not assessed in either pilot or longitudinal studies, a possible feedback mechanism between serum osmolality and circulating prolactin levels leading to depressed plasma prolactin levels after three weeks of potassium adaptation is set forth. It is more likely that chronic stress secondary to high potassium intake induces lower circulating prolactin levels.

This investigation fails to establish a role for prolactin in mediating morphologic and physiologic response of rat ion transporting epithelium to a variety of stimuli including potassium adaptation.



## INTRODUCTION

The pituitary peptide prolactin has been shown to be a major osmoregulatory hormone in lower vertebrates. Prolactin appears to modulate Na-K-ATPase activity in ion transporting epithelium of the euryhaline teleost fish and induce morphologic changes in kidney tissue paralleling the morphologic and physiologic responses observed in rat kidney and colon to a variety of seemingly unrelated stimuli. Such stimuli include chronic potassium load independent of mineralocorticoid, sodium depletion, surgical ablation, and administration of mineralocorticoid or glucocorticoid. Although the osmoregulatory functions of prolactin in higher vertebrates remain unclear, this hormone appears to play a permissive role for other hormonal effects. The possibility that prolactin mediates the common effects of these stimuli in the rat kidney or colon is investigated.

The osmoregulatory effects of prolactin on ion transporting epithelium in lower vertebrates is reviewed along with other hormonal controls of hydromineral balance in the euryhaline teleosts. Effects of prolactin on ion transporting tissue in higher vertebrates is discussed with emphasis on Na-K-ATPase activity. Finally, physiologic and morphologic responses of rat kidney and colon to a variety of stimuli is briefly summarized to suggest a possible common mechanism mediated by prolactin.





## I. OSMOREGULATION IN FISH

### A. The Role of Prolactin in Osmoregulation

The euryhaline teleost fish have the unique capacity for adaptation to marked differences in external salinities and osmotic forces. In the marine environment, the organism must replace water lost to a hypertonic medium and excrete ions. Fresh water environments are a relative hypotonic medium. Hence, the organism must excrete large quantities of water and limit the net loss of ions.<sup>1</sup> This process of osmoregulation occurs at the gill, kidney, skin, intestinal and urinary bladder.<sup>2,3</sup> Alterations in water permeability and ion exchange at these sites is a complex interaction of hormones demonstrating osmoregulatory properties. Early studies of enhanced survival in hypophysectomized euryhaline teleosts following replacement therapy with prolactin emphasized its central role in hydromineral adaptation. Burden demonstrated that the hypophysectomized euryhaline teleost *Fundulus heteroclitus* failed to tolerate fresh water media. Death was associated with hypochloremia, occurring within six to seven days.<sup>5</sup> Replacement therapy with *Fundulus* pituitary brei completely restored survival in this species. These results were confirmed by Pickford, et al<sup>6</sup> who also showed that alternate day injections of naturally occurring cortisol (2.5 and 0.0025 mg/g BW) and aldosterone (24 and 2.4 mg/g BW) failed to promote survival on transfer to fresh water. The smaller doses were considered to be within physiologic range. Whole rat pituitary brei and purified prolactin from *F. heteroclitus* promoted survival to at least twenty days. Later studies by Pickford investigated numerous replacement therapies including adeno and



neurohypophyseal products following hypophysectomy in FW adaptation.<sup>7</sup> Arginine vasopressin (2.25 mU/g BW and 0.0225 mU/g BW) shortened survival time. Similarly, isotocin (the teleostean oxytocin equivalent) injection at 1.32 mU/g BW and 0.0064 mU/g BW shortened survival time. Administration of both hormones increased body weight at time of death by an average 5.4% and 5.6% respectively. Daily injections with 0.06% NaCl and balanced saline solution in similar volumes accelerated failure. Injection of bovine growth hormone 20 mg/g BW three times weekly failed to promote survival although each dose was estimated to contain 8 mU of bovine prolactin. Hypothyroidism after treatment with <sup>131</sup>I did not impair fresh water adaptation of intact *F. heteroclitus*.<sup>8</sup> It was concluded that pituitary preparations were able to promote survival in fresh water after hypophysectomy via prolactin activity.<sup>5,6</sup>

Incomplete hypophysectomy was reported in one fish (*F. heteroclitus*) which was previously noted to survive in fresh water.<sup>7,9</sup> On histologic examination of the pituitary remnant, erythrosinophilic cells in Cleveland Wolfe preparations were found to compose 81% of a mass occupying the rostral pars distalis. The cells demonstrated intracellular features characteristic of the hyperplastic and hypertrophic response after fresh water adaptation, e.g., large round nuclei, prominent nucleoli, and Golgi apparatus, granulated cytoplasm and mitotic figures. Similar cytologic data was reported in stepwise adaptation of the euryhaline cyprinodont *Poecilia latipinna* to changes in external salinity.<sup>10,11</sup> In salt water (SW), cytologic features were consistent with inactivity: cells were small with dark staining kidney-shaped



nuclei, chromatin was condensed, Golgi complex indistinguishable and cytoplasm lacked erythrosinophilic granulation. Fish maintained in 1/3 SW showed increase in cell size ( $P < 0.01$ ) and erythrosinophilic granulation was abundant in most specimens. Other features were essentially unchanged. In fresh water (FW) adapted subjects, cell size was increased three times over those maintained in 1/3 SW. Nucleoplasm was clear and nucleoli were distinct. Golgi complex was abundant as was cytoplasmic RNA. Twelve and thirty hours after transfer from 1/3 SW to FW, cell and nuclear size increased ( $P < 0.001$  and  $P < 0.05$  respectively) nucleoplasm became clear, cytoplasm became degranulated and Golgi complex became more prominent. Concurrently, plasma Na dropped from  $161.8 \pm 3.22$  in 1/3 SW to  $140.6 \pm 3.45$  meq/l after 12 hours and finally  $132.5 \pm 6.45$  meq/l after thirty hours (mean  $\pm$  SEM). In the FW adapted state, plasma Na concentration was  $148.4 \pm 2.71$  meq/l. Prolactin content of pituitary gland was also measured by polyacrylamide electrophoregrams. Content was least in SW, three times higher in 1/3 SW and six times higher in FW. On transfer from 1/3 SW to FW, pituitary prolactin fell significantly to approximately 25% prior to transfer ( $P < 0.01$ ). These observations indicate erythrosinophilic cell secretory response leading to a decrease in pituitary prolactin content concurrent with a rapid fall in plasma sodium. The cellular processes of synthesis and release are initially non-synchronous but by 72 hours granulation reappears and pituitary prolactin content rises to FW adapted levels after equilibration is achieved. This evidence and previous studies suggested that erythrosinophilic cells were the source of endogenous prolactin. Pituitary prolactin





content varied inversely to external salinity after adaptation and indirectly with secretory activity most prominent during adaptation to hypotonic media during which time secretory and release processes are asynchronous. Falling plasma sodium concentration associated with a decline in pituitary prolactin content and erythrosinophil cellular morphology consistent with release and synthesis has also been reported by Clarke.<sup>12</sup>

#### B. Hypophysectomy and Alterations in Osmoregulation

Hypophysectomy is detrimental to the survival of many euryhaline fish after transfer into fresh water. Serum sodium concentration\* falls from  $182.9 \pm 6.3$  meq/l in salt water before hypophysectomy, to  $130 \pm 3.1$  meq/l in FW following hypophysectomy. Serum sodium of intact fish in fresh water was  $172 \pm 4.1$  meq/l. Similarly, serum chloride concentration of intact fish adapted to fresh water was  $126.4 \pm 3.8$  meq/l.<sup>13</sup> There is a simultaneous 40-60% fall in serum osmolarity.<sup>5,14</sup> Administration of exogenous prolactin opposes these changes.<sup>3,13,14,15</sup>

It appears that whole body sodium turnover rate is markedly reduced when the intact euryhaline *P. latipinna* is transferred from hypertonic sea water to hypotonic fresh water. As percent of exchangeable sodium per hour, total sodium flux falls from  $56.7 \pm 3.76\%$  to  $2.2 \pm 0.37\%$  after adaptation (mean  $\pm$  SEM). Hypophysectomy impairs this adjustment such that outflux of sodium is increased from  $1.6 \pm 6.16$  to  $11.8 \pm 3.75$  meq/g/h and net sodium flux increases from -1.0 to

\* all values mean  $\pm$  SEM





$11.5 \pm 3.92$  meq/g/h.<sup>3</sup> This net loss of sodium can be largely prevented by injection of ovine prolactin but not cortisol prior to transfer. Prolactin reduces sodium outflux from  $11.8 \pm 3.75$  to  $2.0 \pm 0.2$  meq/g/h. These studies were carried out on hypophysectomized fish maintained in 1/3 salt water prior to fresh water transfer.<sup>3,15</sup>

### C. Hormonal Control of Gill Osmoregulation

There is a great deal of evidence that Na-K-ATPase mediates ion transport necessary for adaptation to changing external salinities.<sup>16-21</sup> Increased sodium turnover and outflux observed in euryhaline fish adapted to salt water is accompanied by an increase in gill Na-K-ATPase of *Fundulus heteroclitus*.<sup>17,19,21</sup> Enzymatic activity in the gill has been associated with the ionocyte, a unique cell type known to undergo morphologic changes during acclimation to varying salinities.<sup>3,19</sup> Homogenate and microsomal gill Na-K-ATPase from fresh water adapted fish was 3.6 and 12.4  $\mu\text{mole Pi/h/mg}$  protein respectively. In salt water adapted fish, enzyme activity increased to 20.1 and 84.4  $\mu\text{mole Pi/h/mg}$  protein respectively. Enzyme activity of hypophysectomized fish adapted to salt water was reduced to 11.2 and 37.0  $\mu\text{mole Pi/h/gm}$  protein for homogenate and microsomal preparations.

Net sodium uptake increases across the gills of the euryhaline eel *Anguilla anguilla* when maintained in salt depleted media. Similarly, the stenohaline *Cichlosoma biocellatum* (which lives only in fresh water) demonstrates increased ionocyte activity by histologic appearance and activation of pituitary erythrosinophils.<sup>22</sup> The change observed in the ionocytes of the latter fish can be mimicked by injection of mammalian prolactin. In adrenalectomized *Anguilla* main-



tained in fresh water the ratio of sodium influx to external sodium concentration falls from  $33.8 \pm 2.80$  for intact fish to  $19.4 \pm 2.42 \frac{\text{meq/h/g BW}}{\text{meq/l}}$  (mean  $\pm$  SEM,  $P < 0.01$ ). Restoration of sodium balance was observed following administration of cortisol. Hypophysectomy in similar experimental surroundings was accompanied by an apparent increase in sodium outflux.<sup>24</sup> Prolactin restored sodium outflux to normal.<sup>25</sup> It appears that in fresh water prolactin reduces sodium outflux at the gill, an effect observed in other euryhaline species as well.<sup>8</sup> However, prolactin stimulation of the sternohaline (*Cichlosoma biocellatum*) ionocytes in surroundings requiring uptake of electrolytes suggests prolactin has stimulatory effects on active electrolyte absorption mechanisms as well.

Increased activity of microsomal Na-K-ATPase in gills and kidneys has been observed during long term fresh water adaptation of predominantly marine euryhaline species. Later microsomal studies by Gallis, et al<sup>20</sup> confirmed this increase in enzyme activity during long term fresh water adaptation suggesting an increase in electrolyte absorption to hypotonic medium. In short term acclimation to fresh water (five to eight days) Na-K-ATPase activity decreased in gill epithelium from  $12.7 \pm 0.97$  to  $9.4 \pm 1.00 \mu\text{mole P}_i/\text{h/mg protein}$ . Moreover, prolactin (12 UI/100 g BW) decreased enzyme activity during fresh water adaptation from  $9.41 \pm 1.00$  to  $5.5 \pm 0.75$  ( $P < 0.01$ ) and decreased enzyme activity in salt water from  $12.7 \pm 0.97$  to  $7.9 \pm 0.99$  ( $P < 0.01$ , mean  $\pm$  SEM). Since NA-K-ATPase activity increases during salt water acclimation in many euryhaline teleosts accompanied by an increase of sodium efflux across gill epithelium, inhibition of enzyme activity



by prolactin most likely represents a decrease in efflux of sodium during short term fresh water adaptation.<sup>13</sup> This implies either two separate Na-K-ATPase transport mechanisms in the gill epithelium or a single enzyme able to function in opposite directions. Gallis, et al<sup>20</sup> suggests there are indeed two enzyme systems differentiated by inhibition to actinomycin D. Towle, et al, however, demonstrated that Na-K-ATPase from the killifish *Fundulus heteroclitus* was similar with respect to ouabain sensitivity, electrophoretic behavior, cation dependency and response to  $\text{NH}_4$  in both microsomal and homogenate gill preparation from fish acclimated to salt, 16% salt, and fresh water.<sup>26</sup>

Pickford, et al provided evidence to indicate that Na-K-ATPase functions to excrete electrolytes in bronchial epithelium and absorb electrolytes in the kidney in *Fundulus heteroclitus*. Hypophysectomy of salt water adapted fish followed by transfer to fresh water resulted in osmoregulatory failure marked by decline in serum chloride, sodium and osmotic forces. Enzyme activity in homogenate of gill and kidney from hypophysectomized fish three days after transfer to fresh water were  $9.00 \pm 0.60$  and  $12.24 \pm 1.05$   $\mu\text{mole P}_i/\text{hr/g}$  protein respectively. Enzyme activity in fish treated with prolactin 105 mU per g BW for five days during and after transfer showed a fall in gill ATPase to  $3.11 \pm 0.46$  and a rise in kidney activity to  $19.80 \pm 1.32$   $\mu\text{mole P}_i/\text{g/h}$ . Replacement therapy also reversed the fall in serum electrolytes. The fish receiving prolactin remained active without overt signs of lethargy (all values mean  $\pm$  SEM).<sup>13</sup>

#### D. Hormonal Control of Renal Osmoregulation

Renal sodium excretion represents approximately 1/4 to 1/5 of





total sodium outflux and approximately 1/4 of serum sodium concentration in euryhaline teleosts maintained in fresh water.<sup>3,25</sup> Glomerular filtration and urine flow rates are much higher in fresh water adapted compared to salt water adapted fish. In the euryhaline teleost *Fundulus kansae*, GFR in fresh water is 600 ml/kg/day which falls to 2045 ml/kg/day on transfer to salt water (calculations based on inulin clearance). Similarly, urine flow rate declines from 200 mg/kg/day to 5-20 ml/kg/day on transfer to salt water. Values for GFR greater than urine flow rate and  $U_{Na}/P_{Na}$  ratios less than one in fresh water acclimated fish suggests renal tubular reabsorption of sodium,<sup>27,28</sup> Active reabsorption of sodium is supported by the marked increase in Na-K-ATPase activity in kidney homogenate of fresh water adapted *Fundulus heteroclitus* compared to enzyme activity of the salt water adapted fish.

Pituitary ablation in fresh water adapted fish results in increased urine sodium concentration that net renal sodium loss is significantly elevated despite a reduction in urine flow rate from 330 mg/kg/day in control *F. kansae* compared to 220 ml/kg/day in hypophysectomized fish.<sup>23</sup> Prolactin replacement therapy results in decreased urine sodium concentration and increased urinary flow rate such that renal sodium loss remains unchanged or slightly higher compared to intact *F. kansae*.<sup>16</sup> Increase in glomerular tuft size with simultaneous decrease in intracapsular space has been observed following prolactin replacement as well as an increase in the number of glomeruli with decreased intracapsular space implying an increase in both single nephron GFR and total GFR.<sup>3,17,30,31</sup> Prolactin also appears to lower



U/P ratios for sodium and chloride compared to control. This evidence supports reabsorption of an increased filtered electrolyte load.<sup>16</sup> Osmolarity of urine collected from cannulae inflated in the bladder of the starry flounder *Platichthys stellatus* previously injected with ovine prolactin decreased from  $297 \pm 5.3$  mosm/2 in untreated fish to  $193 \pm 18.5$  mosm/2 (mean  $\pm$  SEM,  $P < 0.01$ ). This suggests prolactin mediated reabsorption of urinary osmoles by renal tissue although a simultaneous increase in urine flow rate was observed.<sup>27</sup> Prolactin stimulates renal Na-K-ATPase in *F. heteroclitus* indicating hormonal control of enzyme mediated reabsorption of ions in the kidney of these lower vertebrates.<sup>13</sup>

#### E. Hormonal Control of Urinary Bladder Osmoregulation

Isolated urinary bladder preparations of several euryhaline species have demonstrated osmoregulatory changes responsive to prolactin which parallel physiologic changes during adaptation to fresh water and salt water surroundings. Whereas diffusional permeability remains unchanged in urinary bladders obtained from intact sea water and fresh water adapted flounder, mucosa to serosa permeability  $P_{OS}$  (M-S) fell from  $6.59 \pm 1.73$  in salt water to  $1.81 \pm 0.64 \times 10^4$  cm/sec in fresh water (mean  $\pm$  SEM,  $P < 0.02$ ).<sup>32</sup> Similar reduction in bladder permeabilities have been reported in other euryhaline urinary bladder preparations.<sup>27,33</sup> Bladder preparations obtained from flounder adapted to salt water and treated with prolactin showed a decrease in  $P_{OS}$  (M-S) from  $6.59 \pm 1.73 \times 10^4$  to  $1.34 \pm 0.41 \times 10^4$  cm/sec (mean  $\pm$  SEM,  $P < 0.02$ ). In addition to a less dramatic fall in serosa to mucosa osmotic permeability  $P_{OS}$  (S-M) from  $4.67 \pm 0.77 \times 10^4$  cm/sec (mean  $\pm$



SEM,  $P < 0.05$ ). This represents a reverse in osmotic forces such that the dominance of  $P_{OS}$  (M-S) in untreated salt water preparations is changed to a dominance of  $P_{OS}$  (M-S) in treated preparations. A similar pattern of rectification of flow was observed in the bladder preparation obtained from fresh water flounder although the decrease in  $P_{OS}$  (S-M) was not statistically significant.<sup>32</sup> This pattern of change represents both an overall decrease and directional change in osmotic permeability in fish treated with prolactin and in intact fresh water fish compared to untreated salt water adapted fish. This is compatible with a decrease in reabsorption of water in hypotonic surroundings where the fish must excrete large quantities of water.

Bladder preparations from several salt water acclimated euryhaline teleosts demonstrate increased reabsorption of ions, primarily sodium and chloride after treatment with prolactin. When salt water bladder preparations are compared to preparations pretreated with prolactin, there is a significant rise in mucosa to serosa sodium ion transport in hypotonic half-Ringers solution.<sup>32</sup> Ion transport was completely abolished by addition of ouabain to the preparation.<sup>33</sup> This evidence suggests an active ion transport mechanism activated by prolactin which simultaneously reverses osmotic permeability to water. Hence, prolactin appears to control enzyme mediated absorption of ions and reduces permeability to water favoring fresh water adaptation.

#### F. Hormonal Control of Intestinal Osmoregulation

Utida, et al<sup>34</sup> demonstrated solute linked water flow across intestinal epithelium by ouabain inhibition of in vitro water transport. Previous studies indicated that in salt water adaptation  $P_{OS}$  (S-M)





increased and was greater than that in the opposite direction. Simultaneous increase in mucosa to serosa sodium and chloride flux was also observed. The presence of chloride pump mechanism activated during acclimation to sea water suggested by the development of transmural potential difference (serosa negative) was confirmed in radioisotopic ion flux studies. The development of chloride ion transport mechanism and serosa negative transmural potential difference allows for passive flow of sodium ion in the mucosa to serosa direction along the electrochemical potential gradient. The net transfer of sodium and chloride is approximately equal and in the same direction as net water reabsorption. In fresh water both permeability to water and ions is reduced. Treatment of fresh water adapted fish in vivo with cortisol increased ion and water absorption and increased the serosa negative potential difference. Essentially similar results are observed after administration of ACTH in vivo. Prolactin, however, inhibits the ion transport and permeability to water in salt water adapted eels. This suggests that prolactin inhibits the activity of a chloride pump mechanism and hence acts antagonistically to cortisol in the euryhaline teleost intestine.<sup>33</sup>

#### G. Summary of Prolactin Effects in the Teleost

Prolactin appears to reduce sodium loss in fresh water by reducing outflux across the gill epithelium and perhaps stimulating influx of sodium across the gill in some species. Prolactin increases urinary bladder and apparent renal tubular ion reabsorption. Prolactin also appears to decrease the osmotic permeability of intestinal and urinary bladder membranes. These general effects enable the euryhaline fish





to acclimate to a hypotonic media where retention of ions and excretion of water are essential for survival. These changes in transmembranous movements of ions and water induced by prolactin appear to involve modulation of Na-K-ATPase ion transporting activity.



## II. THE "CHLORIDE CELL" - A MODEL FOR Na-K-ATPase ACTIVITY AND ION TRANSPORT UNDER HORMONAL CONTROL IN LOWER VERTEBRATES

Sodium-potassium dependent ATPase has proven to be ubiquitous in transepithelial transport of ions. The enzyme has been well studied in the teleostean chloride cell or "ionocyte" - the site of branchial osmoregulation required for maintenance of life during acclimation to hypertonic or hypotonic surroundings. The activity of this enzyme in the chloride cell is responsive to changes in salinity and to hormonal manipulations.<sup>1,3,13,16,18,19,21,24,35,36,37</sup>

A parallel rise in sodium efflux from gill epithelium and Na-K-ATPase activity occurs over approximately two weeks following transfer of the fresh water acclimated eel *Anguilla rostrata* to salt water.<sup>37,38</sup> Na-K-ATPase activities in gill homogenate obtained from *Fundulus heteroclitus* acclimated to fresh water, salt water and 200% salt water were  $3.5 \pm 0.03$ ,  $5.7 \pm 0.07$  and  $12.1 \pm 0.9$   $\mu\text{mole P}_i/10 \text{ mg filament/h}$ , (mean  $\pm$  SEM) respectively, demonstrating response to a wide range of salinities.<sup>39</sup> The increase in enzyme activity and efflux of sodium is accompanied by a rise in plasma control that peaks to 10 mg/100 ml approximately two days after transfer of *Anguilla rostrata* from fresh to salt water. This peak occurs in the early stages of increased enzyme activity and closely parallels the transient peak plasma sodium from 160 meq/l to 220 meq/l.<sup>40</sup> Maetz, et al<sup>24</sup> demonstrated cortisol induced increase in gill sodium turnover in the intact eel *Anguilla anguilla* as well as restoration of sodium turnover in the adrenalectomized eel maintained in sea water. Morphologic changes of the chloride cell and the subcellular structure occur in response to changes in salinity and hormonal manipulation. The



chloride cells obtained from branchial epithelium of the pupfish *Cyprinodon variegatus* adapted to 50% salt water, 100% salt water and 200% salt water (SW) did not vary in number. Light micrographs showed no difference in cell size between 50% SW chloride cells and 100% SW chloride cells, although a 50% increase in cell diameter was observed in the 200% SW compared to the 100% SW chloride cell.<sup>42</sup> The number of chloride cells in the silver eel *Anguilla anguilla* acclimated to fresh water (FW) was approximately equal to the number of cells in the SW adapted yellow eel and did not change significantly during SW adaptation. Na-K-ATPase activity increased less than 50% although in vitro microsomal activity was 43.02  $\mu\text{mole P}_i/\text{mg protein/h}$  in SW compared to 26.13  $\mu\text{mole P}_i/\text{mg protein/h}$  of the yellow eel acclimated to fresh water.<sup>43</sup>

Morphologic and physiologic changes in the chloride cell following transfer from fresh to salt water are similar to responses observed in the cortisol treated fresh water adapted eel.<sup>44</sup> In this study, Na-K-ATPase activity of the gill in fresh water increased from  $4.6 \pm 1.2$  to  $12.2 \pm 2.8$   $\mu\text{mole P}_i/\text{mg protein/h}$  (mean  $\pm$  SEM) following injections of 400 mg cortisol/100 g BW/day for several days.<sup>38</sup> Ventral coloration changed from yellow to silver normally observed prior to seaward migration. These changes suggest that cortisol induces morphologic and physiologic changes in gill chloride cells which prepare for an ability to excrete large salt loads.

#### A. Morphologic Changes in the Chloride Cell in Response to Osmoregulatory Stress

The response of the chloride cell to hypertonic surroundings





and cortisol is hypertrophic. A distinct feature of the chloride cell is an extensive tubular system continuous with the basal and lateral cell surfaces demonstrated by horseradish peroxidase and lanthanum tracer studies.<sup>42</sup> Mitochondria are found in high numbers and in close proximity to the tubular system. Longitudinal cross section of the tubules reveals a finger-like branching pattern with branching every 0.1-0.15  $\mu\text{m}$ .<sup>35</sup> The branching pattern becomes more extensive under hypertonic stimulus of 100% and 200% sea water. In fish adapted to 200% sea water, the tubular system proliferates displacing cytoplasm and reducing the space between tubular membrane and mitochondria to approximately 1000 Å. Concurrently the mitochondrial size decreases while density increases accounting for a marked increase in mitochondrial surface area to volume ratio.<sup>35</sup> Amplification of the tubular system was observed following cortisol injection in *Anguilla rostrata*.<sup>44</sup>

Proliferative response of the basolateral invaginations is accompanied by an increase in Na-K-ATPase activity of four fold between 100% and 200% salt water adapted pupfish *Cyprinodon variegatus* paralleling marked increase in sodium turnover.<sup>42</sup> Amplification of the basolateral membrane surface area with simultaneous increase in Na-K-ATPase activity has been observed in the chloride cell of *Fundulus heteroclitus* and *cyprinodon variegatus*,<sup>39,42</sup> in the nasal salt glands of marine birds<sup>46</sup> and in mammalian epithelium demonstrating increased electrolyte transport activity.<sup>45,98,99</sup>

Karnaky, et al<sup>39</sup> demonstrated that the binding of pattern 50  $\mu\text{M}$  ( $\text{H}^3$ ) ouabain perfused vascularly in vivo was similar to the basolateral membrane in *F. heteroclitus* at varying salinities. In-



creased ouabain binding paralleled the increase in Na-K-ATPase observed at higher salinities:  $16.7 \pm 1.0$   $\mu\text{mole/kg}$  filament correlated to ATPase activity of  $13.2$   $\mu\text{mole P}_i/10$  mg filament/h at 200% salt water;  $11.1 \pm$   $\mu\text{mole/kg}$  filament correlated to  $7.3$  ( $n=2$ )  $\mu\text{mole/P}_i/10$  mg filament/h at 100% salt water (mean  $\pm$  SEM). Ouabain perfusion resulted in approximately 80% enzyme inhibition. External irrigation with ( $\text{H}^3$ ) ouabain showed no correlation between binding and increased ATPase activity. Hency, in the chloride cell, the ouabain sensitive Na-K-ATPase known to respond in parallel to proliferation of the basolateral membrane is localized to this same area. In general, ouabain binding is associated with the side of the membrane to which sodium is being transported. It appears, then, that the Na-K-ATPase is oriented to transport Na to the interstitium of the gill.<sup>39</sup> A similar orientation of the membrane associated enzyme exists in mammalian epithelium which demonstrates amplification of the basolateral membrane concurrently with increased Na-K-ATPase function.<sup>45,99</sup>



### III. MORPHOLOGIC CHANGES IN THE EURYHALINE TELEOSTEAN KIDNEY IN RESPONSE TO HORMONAL AND OSMOREGULATORY STRESS

The euryhaline nephron is composed of a glomerulus, a first and second proximal segment followed by a collecting tubule and ureter leading to bladder. Common to the nephron and ureteral epithelium is a well developed paired membrane system occupying the central and lateral cytoplasm. Intramembranous vesicles are abundant. Anastomotic branching is extensive. The membrane system is continuous with the outer cell membrane at the basal and lateral aspects of the cell. Continuity is established through slit-like pores arranged in rows observed more frequently at the cell base. This membrane morphology resembles the tubular system of the chloride cell more closely than the basolateral membranous infolding observed in mammalian ion transporting renal epithelium.<sup>48</sup> In the second proximal tubule the epithelial cell is prismatic without interdigitation of basolateral cytoplasmic processes observed in the proximal convoluted tubule cell in rat kidney.<sup>48</sup> A large population of mitochondria are located near the labyrinth system, a situation similar to that observed in mammalian epithelia. Between 80-90% of total cell mitochondria are located proximal to the labyrinth system with the long axis perpendicular to the cell base.

Freeze fracture studies performed on second proximal tubule cells reveals numerous particles ranging from 8.0 to 12.0 nm in diameter. Four surfaces are created during freeze fracture: outer cell surface (OS), inner cell surface (IS), inner freeze fracture surface (IFF) representing the inner aspect of the cell membrane fractured along



the lipid bilayer and finally the outer freeze fracture surface (OFF) representing the outer aspect of the fractured membrane surface. In the euryhaline three spined stickleback *Gasterosteus aculeatus*, the number of particles observed per  $\text{mm}^2$  was not significantly different when OS of basal labyrinth and outer cell membrane were compared. Similarly, there was no significant difference in particle density on the IS. However, there was an 80% and 40% increase in the particle density in the IFF and OFF respectively when basal labyrinth and outer cell membrane are compared ( $P < 0.001$ ).

TABLE A: Number of particles per unit membrane of IS, IFF, OFF, OS of the basal labyrinth and of the outer cell membranes of the second proximal tubules.

| BASAL LABYRINTH |                                     | OUTER CELL MEMBRANES |                                     |
|-----------------|-------------------------------------|----------------------|-------------------------------------|
|                 | (particles/ $\text{mm}^2$ membrane) |                      | (particles/ $\text{mm}^2$ membrane) |
| IS              | 596 $\pm$ 150                       | IS                   | 595 $\pm$ 45                        |
| IFF             | 3398 $\pm$ 153 *                    | IFF                  | 1844 $\pm$ 135 *                    |
| OFF             | 3789 $\pm$ 124 *                    | OFF                  | 2670 $\pm$ 73 *                     |
| OS              | 1282 $\pm$ 51                       | OS                   | 1212 $\pm$ 82                       |

1. Adapted from Wendelaar Bonga (1974)
2. All values mean SEM
3. \*  $P < 0.001$

Renal tubular reabsorption of monovalent ions occurs during fresh water adaptation in euryhaline teleosts.<sup>16</sup> Bonga, et al demonstrated significant morphologic changes in renal tubular epithelium of the three spined stickleback consistent with this enhanced ion





transporting capacity in fresh water.<sup>47,49</sup> Morphometric studies of ultra thin sections from the second tubule segment comprising approximately 70% of the euryhaline nephron showed a dramatic increase in basal labyrinth membrane surface area per cell volume and per cell ( $P < 0.001$ ) whereas outer membrane surface area remained essentially unchanged. Cell height and width changes were less pronounced.

TABLE B: Morphometric data of the cells of the second proximal tubules of *Gasterosteus aculeatus* adapted to fresh water (FW) and salt water (SW).

|                                                                                       | mean $\pm$ SEM  |                 |
|---------------------------------------------------------------------------------------|-----------------|-----------------|
|                                                                                       | SW              | FW              |
| Cell height ( $\mu\text{m}$ )                                                         | $8.8 \pm 0.5$   | $9.4 \pm 0.6$   |
| Cell width ( $\mu\text{m}$ )                                                          | $3.0 \pm 0.1$   | $3.2 \pm 0.1$   |
| Membrane surface ( $\mu\text{m}^2$ ) of basal labyrinth/<br>$\text{m}^3$ of cytoplasm | $3.5 \pm 0.5^*$ | $6.3 \pm 1.1^*$ |
| Membrane surface ( $\mu\text{m}^2$ ) of basal labyrinth/<br>cell                      | $219 \pm 24^*$  | $466 \pm 57^*$  |
| Surface ( $\mu\text{m}^2$ ) of basal and lateral outer<br>cell membrane per cell      | $93 \pm 4$      | $103 \pm 5$     |

1. Adapted from ref. 49
2. All values mean  $\pm$  SEM
3.  $P < 0.001$

Particle density was quantitated in freeze etch studies of the second proximal renal tubule obtained from sticklebacks adapted to sea water and fresh water. All four freeze fracture surfaces previously described showed significant increases in particle density in outer



cell and basal labyrinth membrane from fresh water adapted tissue ( $P < 0.001$ ) although the increase was less significant for the inner surface.

TABLE C: Percent increase in particle density of second proximal tubule in *Gasterosteus aculeatus* adapted to fresh water compared to salt water.

|     | OUTER CELL MEMBRANE | BASAL LABYRINTH MEMBRANE |
|-----|---------------------|--------------------------|
| IS  | 15% **              | 5% ***                   |
| IFF | 50% *               | 26% *                    |
| OFF | 30%                 | 44% *                    |
| OS  | 45% *               | 60% *                    |

1. Adapted from ref. 49
2.  $P < 0.05$  \*\*\*,  $P < 0.05$  \*\*,  $P < 0.001$  \*
3. Particle density determined by freeze fracture technique

The absolute particle density remains markedly higher in the labyrinth membrane system compared to the outer cell membrane in fresh water adapted fish. Bonga estimates from combined morphometric and freeze fracture data that total particles per cell is 200% greater for the basal labyrinth in fresh water compared to salt water and only 50% greater for the outer cell membrane.<sup>49</sup>

Ericsson and Trump<sup>50</sup> showed in histochemical and physiological studies on euryhaline renal tubular epithelium that membranes of the basal labyrinth system are highly specialized for ion transporting



activity. In the rat, 3.5% of total membrane associated cellular Na-K-ATPase activity was located at the luminal brush border of outer medullary tubular epithelium suggesting a basal or lateral location for most Na-K-ATPase.<sup>51</sup> Further morphologic studies on membrane preparations from outer medullary rabbit kidney epithelium known to contain only Na-K-ATPase membrane associated protein demonstrates the presence of both transmembrane particles and intramembranous particles by freeze fracture and negative staining representing disrupted and intact Na-K-ATPase enzyme units.<sup>51a</sup> The above evidence may be applied to evidence presented by Bonga, et al to suggest that the increased membrane particle density in fresh water adapted killifish represents an increase in membrane associated Na-K-ATPase enzyme units. This is consistent with increase in Na-K-ATPase activity observed in kidney homogenate and microsomal preparations obtained from *Fundulus heteroclitus* acclimated to fresh water.<sup>13</sup>

Fresh water adaptation of euryhaline teleosts depends on monovalent ion reabsorption by renal tubular epithelium.<sup>3,16</sup> Prolactin replacement therapy in hypophysectomized euryhaline teleosts restores kidney function, increases renal Na-K-ATPase activity and permits fresh water adaptation.<sup>3,13,15,16,17</sup> Bonga, et al showed a significant increase in particle density in both outer cell membranes and membranes of the basal labyrinth in freeze fracture studies of second proximal renal tubular epithelium in salt water adapted *Gasterosteus aculeatus* ten hours following acute transfer into fresh water. After twenty hours, particle density approximated fresh water acclimated values for all freeze fracture surfaces. Thus, increase in sodium





reabsorption and Na-K-ATPase activity is accompanied by increase in membrane particle density.<sup>49</sup> Furthermore, injection of ovine prolactin 0.4 IU/gm BW at moment of transfer to fresh water resulted in significant increase in particle density<sup>49</sup> on IFF ( $P < 0.001$ ) and IS ( $P < 0.01$ ) surfaces when compared to untreated transferred fish.

Fresh water adaptation is characterized by an increase in basal labyrinth membrane surface area per cell and per unit of cytoplasmic volume, increased mitochondrial volume in the second proximal tubular collecting duct and ureteral epithelial cells, and increased nuclear size most marked distal to the second proximal tubular epithelium. These changes are completed within ten days after acute transfer to fresh water. Nuclear pore density in second proximal tubular epithelium is increased from  $146 \pm 12$  to  $173 \pm 15$  per  $25 \mu\text{m}^2$  (mean  $\pm$  SEM,  $P < 0.05$ ) ten hours after transfer which approximated fresh water adapted values.<sup>49</sup>

Prolactin has been noted to accelerate the morphometric changes in renal tubular epithelial ultrastructure induced by fresh water adaptation.<sup>47,48,49,52</sup> In *Gasterosteus aculeatus* treated daily with ovine prolactin 0.3 IU/g BW injected intraperitoneally after transfer to fresh water, ultrastructural changes observed during fresh water adaptation were complete by day three. Of particular interest is a 40% increase in basal labyrinth surface area per cell volume by day three in prolactin treated group whereas amplification in membrane surface area was absent in the control group.<sup>52</sup> Bonga, et al proposed that the lag in the control group represents time required for activation of hypophyseal prolactin producing cells and that the



structural changes indicative of increased ion transporting activity occur under the influence of prolactin. Increase in nuclear pores within the first ten hours of fresh water acclimation may indicate an increased rate of nuclear-cytoplasmic interaction related to protein synthesis and perhaps increase in cell membrane particle density.<sup>52</sup> In summary, it appears that prolactin is responsible for inducing morphologic changes in renal tubular epithelium of the euryhaline teleost *Gasterosteus aculeatus* paralleling physiologic changes characteristic of increased ion transporting activity.



#### IV. EFFECTS OF PROLACTIN ON ION TRANSPORTING TISSUES IN HIGHER VERTEBRATES

##### A. Prolactin Receptor Sites in Vertebrate Kidney

Membrane preparations of mammalian and non-mammalian tissues demonstrate specific prolactin binding characteristics.<sup>53,54,55,56</sup> Often tissue ion transporting activity parallels the degree of prolactin binding as in the rabbit mammary gland.<sup>53</sup> Similarly, binding of prolactin to receptor sites in kidney microsomal preparations of the bullfrog *Rana Catesbeiana* emerges during the metamorphosis from aquatic tadpole stage to amphibious stage where hydromineral regulation is transferred from gill structures to the kidney. White, et al demonstrated competitive inhibition and saturation of isotopic ovine prolactin binding to gill and tail microsomal preparations but not to kidney in premetamorphic stages. In later prometamorphosis, prolactin binding to kidney appears and increases throughout metamorphosis to the adult bullfrog as branchial and tail structures regress. Exposure to thyroxine greatly increases renal binding of prolactin in tadpoles which demonstrate accelerated metamorphic changes during thyroxine exposure and in adult bullfrogs. Renal binding of prolactin appears to be specific for prolactin as shown by competitive inhibition of binding by electrophoretically separated bullfrog prolactin.

Specific renal binding of ovine prolactin was demonstrated in the mature male rat by competitive inhibition in microsomal preparations.<sup>57</sup> Furthermore, an absence of inhibition was observed in the presence of ovine LH, FSH and TSH. Ovine GH showed slight inhibition at large high concentrations presumably due to prolactin contamination. Autoradiographic studies of rat kidneys removed after in vivo arterial



infusion of isotopic ovine prolactin demonstrated localization of prolactin to the ascending thick limb of the distal tubule and the collecting duct.<sup>58</sup> Interestingly, adenylate cyclase in medullary homogenate but not cortical homogenate was stimulated by prolactin ( $10^{-4}$  -  $10^{-5}$  M) and ADH (0.5 - 1,000 millipressor units) in this same study. Wallin, et al demonstrated a dose related decrease in urine flow rate associated with an increase in urine osmolality during infusion of both purified rat and synthetic lysine arginine vasopressin in rats with hereditary hypothalamic diabetes insipidus. Urinary cAMP was also noted to increase in a dose related fashion. Prolactin did not alter  $T^C_{H_2O}$  at infusion rates causing a negative free water clearance. Hence, prolactin appears to exert a direct antidiuretic effect.<sup>59</sup> It is unlikely that contamination with ADH was responsible for the observed antidiuretic effect of prolactin since 99% of radioactive ADH tracer separated from both rat and ovine prolactin by column chromatography. Moreover, the eluate fraction corresponding to ADH during purification of prolactin demonstrated no antidiuretic properties. There was no additional effect on medullary adenylate cyclase activity when ADH and prolactin were administered together suggesting that ADH and prolactin bind to the same receptor protein, in which case the prolactin binding site would be less specific than competitive inhibition studies implied. Receptor site masking by either hormone would also explain this lack of additive activity.<sup>58</sup>

Prolactin binding to proximal renal tubular epithelium has been demonstrated by fluorescein-labelled double antibody technique in the rat. Donatsch, et al showed that after in vivo intravenous infusion





of 50 IU of prolactin five minutes prior to removal and rapidly freezing the kidneys, prolactin was localized to the apical border of the proximal tubular epithelium. There was no localization of prolactin in distal nephron segments up to twenty minutes following intravenous prolactin infusion.<sup>66</sup> Solomon, et al demonstrated an antinatriuretic and antidiuretic effect of ovine prolactin infused intravenously at a rate of  $7.1 \mu\text{g/h}/100 \text{ gm BW}$  in anesthetized rats receiving intravenous hypotonic and isotonic water load.<sup>60</sup> Previous in vivo studies have also demonstrated an antinatriuretic effect of ovine and bovine prolactin in the rat, rabbit, and cat.<sup>61,62,65</sup> Since volume expansion with saline infusion results in a natriuresis associated with a reduced absolute and fractional proximal absorption of sodium and an increase in single nephron glomerular filtration rate,<sup>63</sup> Solomon suggested that a decreased response to saline infusion represented an effect of prolactin on proximal tubular function. No significant difference in GFR or RBF was noted in experimental groups when compared to control groups in this study, thus excluding the possibility that prolactin decreases natriuresis and induces antidiuresis through changes in these physiologic parameters. Moreover, intravenous infusion of bovine prolactin in cats and ovine prolactin in dogs have demonstrated a rise in GFR.<sup>61,64</sup> The mechanism of prolactin's effect on proximal tubular function remains obscure. Direct stimulation of renal Na-K-ATPase function by prolactin in the rat has not been demonstrated although such a mechanism has been proposed to account for decreased sodium excretion.<sup>67</sup> The precise location(s) for prolactin to exert the physiologic effects observed in the mammalian kidney



briefly outlined above remains unclear.

As in the bullfrog, thyroxine ( $T_4$ ) significantly elevated renal binding sites above intact control rat kidney homogenate ( $9.3 \pm 0.8\%$  to  $15.6 \pm 1.9\%$ , mean  $\pm$  SEM). Thyroidectomy and hypophysectomy reduced renal ovine prolactin binding which was restored by daily injections of thyroxine 10 mg/100 g BW/d for two to four days.<sup>67</sup>

#### B. Prolactin Mediated Stimulation of Na-K-ATPase in Mammalian Tissues

Although stimulation of renal Na-K-ATPase by prolactin has not been demonstrated in the rat, Lipton, et al have demonstrated an increase in ( $H^3$ ) ouabain binding ( $16 \pm 4.8\%$ ,  $P < 0.002$ ) in guinea pig myometrium incubated in Krebs-Csapo solution containing 10 mg ovine prolactin/ml over a twenty-four hour period. Increase in ouabain binding was also observed in myometrium from ovariectomized guinea pigs pretreated with 100 mg estradiol/kg BW or 100 mg/kg BW medroxyprogesterone four days prior to sacrifice ( $81 \pm 5\%$  and  $23 \pm 6.4\%$ , mean  $\pm$  SEM,  $P < 0.01$ ). Prolactin incubation failed to augment ( $H^3$ ) ouabain binding in estradiol pretreated groups presumably because maximal increase in Na-K-ATPase was observed at this dose of estradiol. Prolactin also failed to augment ( $H^3$ ) ouabain binding in medroxyprogesterone pretreated groups although an increase of  $13 \pm 4.7\%$  (mean  $\pm$  SEM,  $P < 0.05$ ) was observed in estrogen and progesterone pretreated groups. These investigators suggest that since progesterone is known to have inhibitory effects on estrogenic activity, failure to stimulate ouabain binding after treatment with prolactin in the medroxyprogesterone pretreated group may indicate a similar mechanism of progesterone mediated antagonism.<sup>68</sup>



Tissue slices of lactating mammary alveolar tissue incubated in krebs bicarbonate buffer solution for one hour containing concentrations of ovine prolactin ranging from  $4.35 \times 10^{-8}$  to  $17.40 \times 10^{-8}$  M showed a decrease in sodium ion concentration from  $114.1 \pm 2.1$  meq/kg tissue in control tissues compared to  $105 \pm 2.2$  meq/kg tissue in experimental tissues (mean  $\pm$  SEM,  $P < 0.01$ ). Tissue concentration of  $K^+$  ion did not change significantly. Tissue was obtained from post-partum rabbits injected subcutaneously with bromocriptine 1 mg/kg three days prior to sacrifice. Tissue incubation with ouabain and prolactin abolished the decrease in tissue sodium content observed after prolactin incubation alone, increasing sodium content to values obtained for tissue incubated with ouabain alone. This suggested alveolar cell sodium concentration is a function of prolactin modulation of Na-K-ATPase activity.<sup>69</sup> (Table D, following page.)





TABLE D: Monovalent ion concentration in slices of mammary alveolar tissue from rabbits after incubation in the presence or absence of prolactin (43.5 nM = 1.0 mg/ml) and/or ouabain (0.1 mM).

| <u>Incubation medium content</u> | <u>Monovalent ion concentration</u> | <u>Calculated intracellular ion concentration meq/L intra-cellular water</u> | <u>Na<sup>+</sup>/K<sup>+</sup> ratio</u> |
|----------------------------------|-------------------------------------|------------------------------------------------------------------------------|-------------------------------------------|
| Control                          | 107.3 ± 2.2                         | 137.2 ± 6                                                                    | 126.1 ± 3.6                               |
| Prolactin                        | 98.6 ± 1.9*                         | 109.2 ± 5.2*                                                                 | 132.5 ± 4.5*                              |
| Prolactin and ouabain            | 129.4 ± 3.8**                       | 192 ± 9.9**                                                                  | 45.8 ± 2.4**                              |
| Ouabain                          | 133.1 ± 3.1**                       | 204.7 ± 8.9**                                                                | 50.4 ± 2.6**                              |
|                                  |                                     |                                                                              | 4.06**                                    |

1. Adapted from reference 70

2. \* P < 0.05; \*\* P < 0.01

3. Chloride concentrations remain statistically unchanged

4. All values mean ± SEM



In a similar study Falconer, et al later demonstrated a linear relationship between incubation concentration of prolactin (2.2 to 4.35 nM) and decreasing concentration of sodium in whole wet tissue samples ( $100.5 \pm 4.4$  to  $93.2 \pm 3.4$  meq/kg tissue, mean  $\pm$  SEM). In contrast to the first study, both whole tissue and calculated intracellular potassium concentrations increased compared to control:  $46.8 \pm 1.3$  to  $50.5 \pm 1.6$  meq/kg tissue and  $126.1 \pm 3.6$  to  $132 \pm 4.5$  meq/l intracellular fluid (mean  $\pm$  SEM reaching statistical significance of  $P < 0.05$  in both parameters at prolactin concentrations of 43.5 nM).<sup>70</sup> Incubation of lactating mammary tissue in solution containing prolactin and ouabain or ouabain alone increased tissue and intracellular sodium concentrations while decreasing tissue and intracellular potassium concentrations.

The intracellular  $\text{Na}^+/\text{K}^+$  showed a significant decrease with prolactin and/or 20 nmole ouabain in mammary tissue of pseudo-pregnant rabbits. Since Na-K-ATPase and prolactin receptors have been located in the basal region of mammary alveolar cells,<sup>71,72</sup> these ionic shifts induced by prolactin and blocked by ouabain present strong evidence for the stimulation of Na-K-ATPase enzyme by prolactin.

The mechanism of activation of Na-K-ATPase by prolactin in mammalian epithelial tissue remains unclear. Lipton, et al have presented evidence for an increase in number of enzyme units.<sup>68</sup> However, the time interval allowed (one hour) for the observed changes in ion concentrations in rabbit mammary tissue suggests activation of the membrane associated enzyme units.<sup>69,70</sup> As previously discussed, mammalian prolactin has been shown to increase renal Na-K-ATPase



function in the killifish *Fundulus heteroclitus*.<sup>13</sup> Moreover, prolactin accelerates morphologic changes including basal labyrinth surface particle density known to occur in kidney during fresh water adaptation of the three spined stickleback *Gasterosteus aculeatus*. Increase in membrane particle density occurs within ten hours after prolactin treatment.<sup>49,52</sup> Association between membrane particles and membrane bound Na-K-ATPase suggests rapid synthesis of these enzyme units. Guinea pig myometrium incubated in prolactin medium over twenty-four hours shows an increase in Na-K-ATPase as indicated by ( $H^3$ ) ouabain binding suggesting an increase in enzyme units.<sup>68</sup> Rillema and co-workers have shown an increase in membrane associated phospholipase A by measuring increases in arachidonic acid concentration vs. phosphatidylcholine in in vitro mid-pregnancy mouse mammary gland membrane preparations. This may account for rapid changes in Na-K-ATPase function in mouse mammary tissue since changes in lipid composition of cell membranes, which might occur secondary to activation of phospholipase A and release of arachidonic acid, is known to effect Na-K-ATPase function.<sup>73,74</sup>

In summary, there appears to be a prolactin mediated activation of Na-K-ATPase in mammalian tissues related to increased activity with or without synthesis of additional enzyme units. It is unclear whether this increase in Na-K-ATPase units is due to an increase in membrane surface area containing Na-K-ATPase, as is observed in the chloride cell of the pupfish *Cyprinodon variegatus* and in the eel *Anguilla anguilla*, and in the renal tubular epithelium of the three spined stickleback in response to osmoregulatory stress or to prolac-



tin.<sup>42,44,48,49,52</sup> Increase in particle density and concurrent increase in membrane surface area of the basal labyrinth occurs in response to prolactin in the latter euryhaline teleost suggesting that both membrane surface amplification and increased membrane Na-K-ATPase enzyme density occurs.





## V. PHYSIOLOGIC AND MORPHOLOGIC CHANGES OF MAMMALIAN ION TRANSPORTING EPITHELIUM

### A. Potassium Adaptation

Adaptive physiologic and morphologic changes observed in rat renal tubular epithelium and colonic mucosa during chronic and acute osmoregulatory stress has led to questions concerning the mechanisms underlying the regulation of Na-K-ATPase.

The precise mechanisms responsible for regulating ionic gradients in the rat nephron and colonic mucosa are not completely understood. However, the distal tubular collecting duct and colonis epithelium share several basic physiologic properties; e.g., they appear to have low ion conductivity and possess the ability to secrete potassium ions.<sup>75,76</sup>

Micropuncture techniques (ref. 75, review) have provided firm evidence that greater than 90% of the filtered potassium load is re-absorbed in the proximal tubule. Tubular fluid arriving at the distal tubule is virtually devoid of potassium and hence urinary potassium content is a function of distal nephron epithelial capacity to secrete potassium. Earlier work by Alexander and Levinsky showed that rats maintained on diet containing 10% KCl for 4-6 days maintained lower plasma potassium values compared to controls two hours after an intra-peritoneal injection of 5  $\mu$ mole/kg body weight KCl ( $4.3 \pm 0.5$  vs.  $5.0 \pm 0.05$  meq/l; mean  $\pm$  SEM,  $P < 0.01$ ). Wright, et al<sup>96</sup> showed that in rats maintained on high potassium and low sodium diets, fractional excretion of filtered  $K^+$  was significantly higher than in control animals during acute intravenous infusion of KCl although fractional excretion increased in all animals. Linear regression analysis of K/IN (TF/P)



ratio vs. distal tubule length showed a significant increase in slope only in rats previously adapted to high K diet ( $P < 0.001$ ), although the ratios for both low Na and high K adapted rats after approximately 80% distal tubule length was significantly higher than in rats not receiving high K infusion. The amount of potassium leaving the distal tubule in the K loaded group was approximately 150% of the filtered quantity whereas in the Na depleted group, this was only 75% compared to 55% in normal rats. Taken together with final fractional potassium excretion, this data suggested that approximately 40% of excreted potassium was secreted by the collecting tubule epithelium during infusion of KCl in the Na depleted group. Recently Hayslett, et al suggested that net absorption and net secretion of potassium in the medullary collecting duct paralleled the need to conserve or excrete potassium. Rats maintained on a K depleted diet for 72 hours demonstrated a fall in K/IN (TF/P) ratio of 2.2% to 0.2% from the beginning of the inner medullary collecting duct to final urine. Conversely, in K repleted rats acutely infused with KCl this fractional excretion increased from 12% to 21% along the same collecting duct segment, representing 43% of K excreted in the urine.<sup>84</sup>

#### B. Na-K-ATPase: Role in Potassium Secretion

Micropuncture studies of the rat distal convoluted tubule has demonstrated an increase in the transepithelial potential difference ( $PD_{TE}$ ) in rats fed a high K diet from 58.8  $\pm$  3.4 mv to 75.2  $\pm$  4.8 mv (mean  $\pm$  SEM) compared to control group on normal diet. Wright, et al suggested this increase in  $PD_{TE}$  might arise from a higher peritubular membrane potential difference.<sup>96</sup> Moreover, since plasma K remained



unchanged in the K loaded animals and relatively higher permeability of the peritubular membrane to K is believed to give rise to a diffusion potential, then intracellular K concentration is increased.<sup>75</sup> Although the precise intracellular concentration of potassium remains uncertain, the potential difference across the peritubular membrane and the higher intracellular than extracellular concentration of potassium has suggested the presence of an active transport mechanism for potassium at the peritubular membrane. Unidirectional flux measurements using  $K^{42}$  has confirmed the major role of the peritubular membrane in regulating the transepithelial transport of potassium. Rats maintained on a high potassium diet have increased net fluxes of sodium across the luminal membrane and the peritubular membrane paralleled by an increase in intracellular concentration of labelled potassium in rat distal tubular epithelium.<sup>78</sup> This evidence suggests that potassium secretion occurs as a transcellular process in the distal tubule.

The presence of a Na-K-ATPase pump mechanism in the basolateral membrane exists in both rat distal nephron and colonic mucosa. Characterization of this enzyme's activity under conditions of increased potassium secretion suggested that it is an important mechanism in the regulation of transepithelial potassium movement.<sup>79,80,81,82,83,85</sup> Silva, et al demonstrated an increase in outer medullary Na-K-ATPase activity of 30% ( $P < 0.01$ ) in rats maintained on an average potassium intake of 4.5 meq/100 g BW/d compared to control intake of approximately 1.3 meq/100 g BW/d for seven days. Higher potassium intake of approximately 20 meq/100 g/day induced an increase in the cortical





Na-K-ATPase of 27% ( $P < 0.01$ ) after seven days and an increase of 58% ( $P < 0.01$ ) after 14 days as well as an increase in medullary Na-K-ATPase of 44% after 14 days. Serum potassium concentration in the potassium loaded rats was not significantly higher than in control rats.<sup>79</sup> In a more recent study, Finkelstein, et al demonstrated a 44% increase ( $P < 0.05$ ) in the inner medullary Na-K-ATPase in rats receiving a similar high potassium intake.<sup>85</sup> During infusion of KCl, maximal urinary potassium excretion (40% > control,  $P < 0.01$ ) paralleled this increase in Na-K-ATPase observed in both cortical and medullary tissue from rats maintained on a high K diet.

Increase in renal Na-K-ATPase activity was also observed in renal insufficiency induced by 75% reduction in renal mass in the rat. In kidney homogenate obtained from rats fed normal diets ten to fourteen days following 75% reduction of renal mass, cortical and outer medullary Na-K-ATPase activity rose approximately 20-25%. Although in vivo inulin clearance decreased from  $965 \pm 54$  to  $383 \pm 32$  ml/min per 100 g BW after reduction in renal mass ( $C_{IN}$  remained unchanged with respect to kidney wt.), fractional excretion of potassium was three times higher compared to control rats. During infusion of 0.5 M KCl at 0.02 ml/min, increased fractional excretion of potassium was approximately twice the increase observed in control rats.<sup>80</sup>

### C. Specificity of Na-K-ATPase Activity

As previously discussed, transport of potassium across the peritubular membrane is thought to be related to the net transepithelial movement of K ions.<sup>78</sup> In light of this concept it is important to point out that the activity of other marker enzymes along the baso-



lateral (peritubular) membrane do not change in either chronic potassium loading or in surgically induced renal insufficiency while Na-K-ATPase rises. In both conditions the specific activity of 5' nucleotidase and Mg-ATPase remained unchanged. Furthermore, the increase in Na-K-ATPase during these conditions of increased potassium secretion has been restricted to kidney and colonic tissue: Na-K-ATPase activity homogenates and microsomal preparations of brain, liver and muscle have remained unchanged.<sup>79</sup> Increase in Na-K-ATPase appears to be a specific phenomenon limited to the mammalian kidney and colon in response to potassium adaptation and renal insufficiency.

#### D. Role of Aldosterone in Potassium Adaptation

Increase in renal potassium secretion and Na-K-ATPase during potassium adaptation appears to be independent of the mineralocorticoid effect of aldosterone.<sup>79,81</sup> Aldosterone secretion is known to rise in response to low serum sodium and high serum potassium.<sup>86</sup> However, rats maintained on a sodium depleted diet for seven days did not demonstrate an elevation in renal Na-K-ATPase activity. Moreover, adrenalectomized rats receiving deoxycorticosterone acetate (DOCA) 0.05 mg IM daily as mineralocorticoid supplement and maintained on high potassium diet for seven days demonstrated an increase in both cortical and outer medullary Na-K-ATPase.<sup>79</sup> This dose of DOCA has no effect on renal Na-K-ATPase in adrenalectomized rats.<sup>81</sup> Similarly, colonic mucosa from adrenalectomized potassium adapted rats maintained on 0.05 mg DOCA IM injection daily demonstrated an increase in Na-K-ATPase from  $3.2 \pm 0.6 \mu\text{mole P}_i/\text{mg protein/h}$  to  $5.0 \pm 0.05 \mu\text{m P}_i/\text{mg protein/h}$  (mean  $\pm$  SEM,  $P < 0.05$ ). Furthermore, potassium secretion and transmural



potential difference paralleled this increase in Na-K-ATPase activity. Although this evidence suggests that changes observed during chronic potassium loading is independent of aldosterone or mineralocorticoid effect, adrenalectomy in the rat resulted in a significant fall in Na-K-ATPase, transepithelial potential difference and potassium secretion ( $P < 0.01$ ) in rat colonic epithelium.<sup>79</sup>

#### E. Effect of Glucocorticoid and Mineralocorticoid on Ion Transporting Epithelium

The precise role of mineralocorticoids and glucocorticoids in potassium secretion remains unclear although they have been shown to increase Na-K-ATPase activity, transepithelial potential difference, and potassium secretion in physiologic and/or pharmacologic doses in the mammalian colonic or distal nephron epithelium. Pharmacologic doses of the mineralocorticoid deoxycorticosterone acetate (DOCA) 0.5 mg/100 g BW/day SC for seven days in normal and in adrenalectomized rats significantly increased renal Na-K-ATPase in cortical homogenate but not in the outer medulla when compared to controls.<sup>87</sup> Intact rabbits injected daily for 11 to 18 days with 5 mg DOCA demonstrated an increase in potassium secretion and transepithelial potential difference six times the control group values in the collecting tubule.<sup>92</sup> Aldosterone 10 mg and 100 mg/100 BW/day failed to change renal Na-K-ATPase in intact rats but increased the outer medullary Na-K-ATPase activity to near normal levels in adrenalectomized rats when compared to intact controls. The synthetic glucocorticoid methylprednisolone 3 mg/100 g BW/d SC for three days increased the activity of Na-K-ATPase in both cortical and outer medullary homogenate of rat kidney. In-





terestingly, the enhancing effect of this glucocorticoid was not inhibited by spironolactone 14 mg/100 g/day given subcutaneously for seven days. The increased activity in Na-K-ATPase observed following mineralocorticoid (either DOCA or aldosterone) was inhibited by spironolactone.<sup>87</sup> The differential response between mineralocorticoid and glucocorticoid indicate differences in cell receptors for these two classes of steroid hormones.

Although aldosterone is known to effect electrolyte transport in response to changing levels of sodium or potassium, the roles of both mineralocorticoid and glucocorticoid in maintaining basal mammalian epithelial ion transport is unclear. Bastl, et al<sup>88</sup> recently demonstrated that aldosterone administered in an approximate physiologic dose (10 mg/100 mg BW/day) failed to restore transmural potential difference and potassium secretion in rat colonic mucosa following adrenalectomy compared to control intact rats. Three times the physiologic replacement dose of aldosterone increased K secretion and transmural potential difference compared to adrenalectomized rats without steroid replacement ( $P < 0.01$ ) and restored these parameters to values for intact control rats. Dexamethasone, considered to exert no mineralocorticoid effects, and methylprednisolone in pharmacologic doses increased potassium movement, specific activity of Na-K-ATPase and transmural potential difference in the colon of intact rats.<sup>90,91</sup> Bastl, et al showed that at physiologic doses of 10 mg/100 g BW/day, dexamethasone restored potassium secretion and transmural potential difference to intact values in adrenalectomized rats.<sup>88</sup> Feldman, et al<sup>93</sup> demonstrated less than 2% binding of glucocorticoid to aldo-





sterone receptor sites in rat kidney. This evidence suggests that as in the rat distal nephron, the physiologic changes induced by both mineralocorticoid and glucocorticoid are mediated at distinct sites in the rat colon.<sup>88</sup>

One might speculate that similar physiologic changes observed as variable responses to differing classes of adrenal steroids as well as chronic potassium adaptation and surgical ablation in these mammalian systems are mediated through a common mechanism involving prolactin.



# VI. ENZYME KINETIC PARAMETERS OF RAT COLONIC Na-K-ATPase: EVIDENCE FOR AN INCREASE IN NUMBER OF ENZYME UNITS

Chronic adaptation to a diet containing a high potassium content, treatment with glucocorticoids, and chronic renal insufficiency results in an increase in specific activity of Na-K-ATPase paralleled by an increase in transmural potential difference and potassium secretion in the rat colon.<sup>82,94</sup> Similarly, adaptation to a sodium free diet increases Na-K-ATPase activity and potassium secretion in the rat colon.<sup>94</sup> Recent studies by Kliger, et al<sup>95</sup> have suggested increased active potassium secretion in the distal colon. Active transport processes may be mediated by the Na-K-ATPase enzyme located in the basolateral membrane. Fischer, et al<sup>82</sup> demonstrated that the  $K_m$  for Na-K-ATPase activity in membrane rich fractions of colonic epithelium from potassium adapted rats was not significantly different from control ( $1.1 \pm 0.06$  vs.  $1.1 \pm 0.08$  mM, mean  $\pm$  SEM) where as  $V_{max}$  rose dramatically.  $65.3 \pm 9.5$  vs.  $21.5 \pm 5.5$   $\mu\text{mole}/P_i/\text{mg protein}/h$  (mean  $\pm$  SEM,  $P < 0.05$ ). Similarly, Hayslett, et al<sup>94</sup> have shown that  $K_m$  remains unchanged in sodium depleted rats whereas  $V_{max}$  rose dramatically. In the same study ( $H^3$ ) ouabain binding to colonic Na-K-ATPase plasma membrane rich fractions from potassium adapted rats increased  $38 \pm 9\%$  compared to control and was paralleled by a rise of approximately  $40 \pm 10\%$  in Na-K-ATPase activity. In sodium deprived animals, ( $H^3$ ) ouabain binding and Na-K-ATPase both increased four to five fold. Scatchard plot analysis demonstrated an increase in ouabain binding sites from 53 pmoles/mg protein to 106 pmoles/mg protein in Na-K-ATPase plasma membrane rich fractions after Na deprivation. This evidence taken together indicates an increase in the actual number of Na-K-ATPase pump sites accounting



for the increase in Na-K-ATPase specific activity in rat colon subject to stresses associated with increased potassium secretory capacity.<sup>45</sup> Hence, a morphologic change at the cellular level appears to parallel the adaptive response to changes shown to increase Na-K-ATPase specific activity. A similar effect has been observed in mammalian ion transporting epithelium (guinea pig myometrium) and in the renal tubular epithelium of euryhaline teleost fish in response to prolactin.<sup>49,52,68</sup>





## VII. MEMBRANE AMPLIFICATION IN MAMMALIAN ION TRANSPORTING EPITHELIUM

The increased Na-K-ATPase specific activity and potassium secretion previously discussed has led to direct examination of the morphologic changes paralleling these physiologic changes. Wright, et al<sup>96</sup> and Giebisch<sup>75</sup> suggested the collecting duct epithelium in the rat distal nephron was capable of potassium secretion. Finkelstein, et al demonstrated a significantly increased Na-K-ATPase activity in medullary kidney tissue homogenate obtained from potassium adapted rats.<sup>85</sup> Recently, Schon, et al<sup>87</sup> demonstrated an increase in absolute potassium secretion in the medullary collecting duct in potassium adapted rats during acute potassium load from  $617 \pm 47$  to  $1456 \pm 195$  meq/min/100 g BW (mean  $\pm$  SEM,  $P < 0.05$ ). Kinetic studies on Na-K-ATPase indicated an increase in the number of Na-K-ATPase units in colon mucosal membrane fractions in potassium adapted rats.<sup>94</sup> Stereologic analysis on electron micrographs taken of rat outer medullary collecting duct showed a 32% increase of the basolateral membrane surface density  $S_{VBLM}$  in the principal cell population of potassium adapted rats compared to control,  $3.04 \pm 0.10$  vs.  $2.31 \pm 0.16$   $M^2/M^3$  (mean  $\pm$  SEM,  $P < 0.005$ ). The density ratio of basolateral surface area to tubular basement membrane in principal cells of potassium adapted rats compared to control were significantly higher,  $8.29 \pm 0.62$  vs.  $5.69 \pm 0.37$   $M^2/M^2$  (mean  $\pm$  SEM,  $P < 0.005$ ). Principal cell area and luminal surface density were not significantly different between control and experimental animals. The close correlation of 32% increase in basolateral membrane surface density and 35% increase in outer medullary Na-K-ATPase activity suggests an increase in number of Na-K-ATPase



enzyme units.<sup>85,98,99</sup>

Wade, et al<sup>89</sup> demonstrated basolateral membrane amplification in principal cells of the cortical collecting duct of rabbits 11-18 days following daily IM injections of either mineralocorticoid (DOCA, 5 mg/day) or glucocorticoid (dexamethasone, 5 mg/d)  $Sv_{BLM}$  increased from  $3.01 \pm 0.29$  to  $5.17 \pm 0.45$  in the DOCA treated group (mean  $\pm$  SEM,  $P < 0.01$ ). These morphologic changes were paralleled by significant increases in  $PD_{TE}$  ( $P < 0.01$ ). O'Neil, et al<sup>92</sup> showed that after 11-18 days at the same dose of DOCA, potassium excretion increased nearly six times.

Stereologic analysis of rat ascending and descending mucosal colonic epithelium in potassium adapted, sodium deprived and dexamethasone treated rats (conditions known to increase potassium secretion, Na-K-ATPase activity transmural potential difference and ouabain binding in colonic epithelium)<sup>82,88,91,94</sup> was performed to assess changes in basolateral membrane surface density. In potassium adapted and Na deprived rats,  $Sv_{BLM}$  increased significantly in both ascending and descending colon ( $P < 0.05$ ). In rats treated with a pharmacologic dose of dexamethasone 600 mg/100 gm BW/day<sup>91</sup> for three days  $Sv_{BLM}$  increased significantly only in the descending colon ( $P < 0.05$ ). The ratio of  $Sv_{BLM}$  to length of basement membrane was increased in the potassium adapted and sodium deprived animals. Apical surface density and cell dimensions were not different between experimental and control groups.<sup>99</sup> These results suggest that the increased potassium excretion, ATPase activity, and ouabain binding may be a result of increased membrane surface area in the basolateral membrane and hence an increase in



available Na-K-ATPase pump sites thought to contribute to potassium secretion. Whether there is an increase in pump site density remains uncertain.



# VIII. PURPOSE OF THIS STUDY

Changes in transporting membrane surface area and ion movement have been observed in lower vertebrates. Maintenance of hydromineral balance during ionic and osmotic stress is mediated by prolactin in the euryhaline teleost *Fundulus heteroclitus*. This preservation is accompanied by a rise in renal ATPase activity. In the three spined stickleback *Gasterosteus aculeatus*, prolactin has been shown to be responsible for preservation of osmoregulation during changes in external salinity and induce morphologic changes in the renal tubule epithelium similar to the changes observed in mammalian ion transporting epithelium adapting to various manipulations which cause increased transepithelial ion movement and Na-K-ATPase activity. Mammalian ion transporting epithelium also appears to respond to prolactin. ( $H^3$ ) ouabain binding increases in guinea pig myometrium treated with prolactin. Changes in ionic composition of whole lactating mammary tissue as well as calculated intracellular concentrations in the rabbit are thought to reflect prolactin control of Na-K-ATPase activity.

Physiologic changes in ion transport, Na-K-ATPase activity, transepithelial and transmural potential differences parallel morphologic changes in the basolateral membrane of rat ion transporting epithelium. Prolactin may mediate these physiologic responses to various stimuli as suggested by its effect on ion transport in lower vertebrate and mammalian transporting epithelium.





## MATERIALS AND METHODS

The possibility that prolactin mediates the morphologic and physiologic changes described during potassium adaptation was investigated by measuring serum and plasma prolactin levels during adaptation to an oral diet high in potassium chloride.

### I. EXPERIMENTAL SUBJECTS

Adolescent Sprague Dawley male rats obtained from Camm Laboratory animal suppliers were used in all experiments. In the pilot study, experimental animals weighed approximately 300g at the time of sacrifice twenty days after starting high potassium diet. Control animals weighed approximately 250 g at the time of sacrifice five days after starting the control diet. In the longitudinal study, experimental animal weight remained constant at about 125 g whereas control group weighed approximately 225 g at the time of sacrifice twenty-one days after the start of the study. In the final study, both control and experimental animals weighed approximately 250 g at the time of sacrifice.

Animals in the pilot study and longitudinal study were housed two to a cage. In the third study, animals were housed singly. Lighting in the animal room was on a constant schedule: 0700 to 1900 hours light and 1900 to 0700 hours dark. Ambient temperature was constant at  $26 \pm 2^{\circ} \text{C}$ .

Control animals in the pilot study were fed a food mixture containing sucrose, 48.4%; casein, 30%; lard, 10%; corn oil 5%; vitamin diet fortification mixture, 2%; normal mineral mixture 3% KCl, 1%; and NaCl, 0.6% ( $\text{K}^{+} = 0.13 \text{ meq/g}$  and  $\text{Na}^{+} = 0.1 \text{ meq/g}$  of diet). Experimental



animals were fed a similar food mixture except  $K^+ = 1.74$  meq/g. Daily intake varied between 10 to 15 g for both experimental and control groups. All control rats in the longitudinal and third study were given powdered Rodent Laboratory Chow #5001 containing 0.14 meq  $K^+$ /gm. Experimental rats were fed the same powdered diet containing 2.14 meq  $K^+$ /gm. Daily intake varied between 15 to 20 g for experimental and control groups. Animals in all groups were offered tap water ad lib. All animals were fasted in the longitudinal and third study six hours prior to sacrifice since oral food intake has been associated with elevated serum prolactin levels in humans.<sup>94</sup>

All animals were sacrificed by rapid decapitation and trunk blood collected in centrifuge tubes on ice, fitted with individual funnels. Since acute stress has been shown to elevate serum prolactin levels in the rat,<sup>101</sup> decapitation was chosen as the blood collecting method. Animals were not anesthetized prior to sacrifice since plasma levels have also been noted to rise 3-4 fold within two minutes after exposure to ether anesthesia.<sup>102</sup> Animals were sacrificed in a separate room within thirty seconds of removal from cage except in the pilot study where decapitation was performed in the same room. In the pilot studies, time of sacrifice was 1100 and 1500 hours. This time interval represents a nadir circadian pattern of serum prolactin. In the third group, animals were sacrificed just prior to the dark cycle. Several studies have shown rising plasma prolactin levels in the late afternoon.<sup>105,106</sup> A peak in the circadian pattern of plasma prolactin concentration has been noted just prior to the dark cycle although the peak has been attributed to anticipation of feeding.<sup>107</sup>



Between 1 to 3 ml of plasma (pilot and longitudinal study) or serum (third study) per rat was collected after refrigerated centrifugation at 1600 RCF units for 20 minutes. The samples were stored at  $-5^{\circ}\text{C}$  for at most two weeks until radioimmunoassay was performed.





## II. RADIOIMMUNOASSAY

Reagents to perform radioimmunoassay (RIA) for rat prolactin were obtained from the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD). Rat prolactin reference preparation (NIAMDD-rPRL-RP-2, AFP-2281B) was supplied in 1% BSA lyophilized form. Rat prolactin antiserum (rabbit, NIAMDD-anti-rPRL-5-8) was supplied in 2% normal rabbit serum. Both immuno-reagents were divided into aliquots and stored at  $-5^{\circ}\text{C}$ . NIAMDD rat prolactin radioimmunoassay is specific for prolactin at the concentrations studied as determined by Neill, et al.<sup>108</sup> Radiolabelled rat prolactin was obtained from New England Nuclear as prolactin ( $\text{I}^{125}$ ) containing between 1 to 5% unbound iodide. Only "hot" prolactin iodinated within one month was used for any radioimmunoassay in this investigation. Precipitation of primary antibody-antigen ( $1^{\circ}\text{Ab-Ag}$ ) complex was performed using the secondary antibody technique. Goat anti-rabbit Gamma Globulin (GARGG) was obtained from CalBiochem initially in 125 U aliquots and then in bulk form of 4500 U. GARGG was stored at  $-5^{\circ}$  in appropriate aliquots to prevent repetitive freeze-thawing.

Each "pot" reaction mixture contained 1/3 3% NRS in normal saline 0.1 M phosphate buffer with EDTA (0.1 M) at pH 7.6 and 2/3 1% BSA in normal saline phosphate buffer with EDTA at pH 7.6. Radiolabelled prolactin was added in sufficient quantity such that total cpm in reaction vials was between 10,000 and 15,000. Nonspecific binding by secondary antibody was determined for each assay by transferring an appropriate amount of "pot" reaction mixture containing only NRS, BSA and radiolabelled prolactin. Primary antiserum was added to the



reaction mixture such that dilution in final reaction vial was 1:10,000. The reaction mixture was incubated for twenty-four hours. The maximum binding dilution of primary antiserum at  $24 \pm 2^{\circ} \text{C}$  was determined by titrating serial dilutions against radiolabelled prolactin.

Reference prolactin RP-2 dilutions were prepared by using 1% BSA (Sigma, RIA Grade) in normal saline phosphate buffer as diluent in serial dilutions of 0.5 ng, 1.0 ng, 2.0 ng, 4.0 ng, 8.0 ng, and 10.0 ng per ml of buffer. Two hundred  $\mu\text{l}$  of standard RP-2 dilutions were added to final vials containing 600  $\mu\text{l}$  reaction mixture. Reference vials (Ref.) contained 200  $\mu\text{l}$  1% BSA normal saline buffer in place of standard prolactin antigen. Vials labelled NSB, Ref, and dilutions of standard prolactin were run in replicate for each standard curve.

Dilutions of 1:2, 1:4, and 1:8 were performed on rat serum or plasma in a volume of 200  $\mu\text{l}$  using 1% BSA normal saline buffer with EDTA ( $\text{pH} = 7.6$ ) as diluent. Dilutions were performed in individual vials containing 600  $\mu\text{l}$  of the pot incubated at  $24 \pm 2^{\circ} \text{C}$  for twenty-four hours. Replicate dilutions were performed.

Secondary antibody (GARGG) was titrated by serial dilutions to maximize binding of primary Ab-Ag complex for each lot of secondary antibody used in the study. Two hundred  $\mu\text{l}$  of GARGG at appropriate dilution was added to each vial. Final reaction volume was 1 ml.

After an incubation period of twenty-four hours at  $24 \pm 2^{\circ} \text{C}$ , vials were centrifuged ( $\text{Temp} = 5^{\circ} \text{C}$ ) at approximately 1600 RCF units for thirty minutes. A Beckman (Gamma 4000) gamma counter was used



to count both decanted supernatant and precipitate.

Pooled plasma from six control rats was used to determine dilutional linearity. This same pooled plasma was included in all assays in the longitudinal study to assess inter-assay variation and linearity of plasma dilution with respect to plasma prolactin concentration.



### III. EXPERIMENTAL DESIGN OF STUDIES

#### A. Pilot Study

Six rats were fed a high potassium lard diet for twenty-one days. The amount of food consumed ranged from 10 to 15 g daily. Control rats were introduced five days prior to completion of the three week period of rat potassium adaptation and were fed control lard diet. Rats were sacrificed at approximately 1100 hours by decapitation alternating between pairs of experimental and control rats. Decapitation was performed in the same room in which the animals were housed.

#### B. Longitudinal Study

Thirty-six rats were fed a high potassium Purina powder diet and thirteen control rats were fed unaltered Purina powdered diet. All rats were housed in pairs except for three rats housed together for nine days and then as pairs from day twenty-two of potassium adaptation until sacrifice. Food intake averaged 15-20 g powder diet per rat per day. All rats were fasted before sacrifice at 1500 hours. Each rat was decapitated in a separate room. Care was taken not to startle the animal. Materials used to sacrifice the animals were thoroughly rinsed between decapitations.

All rats were allowed to acclimate to the housing environment for approximately five days before the start of potassium adaptation. Control rats were sacrificed on day one (six rats), day nine (three rats) and day twenty-two (four rats). Experimental rats were sacrificed on day three (six rats), day five (six rats), day nine (six rats), day twelve (six rats), day seventeen (six rats) and day twenty-two (four rats).





### C. Third Study

Nineteen rats were used to assess serum prolactin levels in potassium adapted rats during a rise in the circadian pattern of serum prolactin in the rat. All rats were housed singly and allowed to acclimate to the experimental surroundings for five days prior to the start of potassium adaptation. During this time all rats were fed unaltered Purina powdered food. At 1800 hrs of the first day of potassium adaptation, six control rats were sacrificed. The daily dietary intake of the remaining six control and seven experimental rats averaged 15-20 g powder diet per rat per day. Morphologic changes in rat kidney observed after potassium adaptation occurs within five days.<sup>103</sup> Following this time period, both experimental and control rats were sacrificed at 1800 hours. All rats were sacrificed in the same manner as in the longitudinal study.

Serum osmolality was determined for each rat by freezing point depression.



#### IV. METHODS OF DATA DERIVATIONS AND STATISTICAL ANALYSIS

##### A. Standard Curve

Original data generated from counting gamma particle emission in the precipitate and supernatant of all vials having completed incubation periods required for successful competitive inhibition are referred to as "bound" and "free" values respectively. Nonspecific binding of the secondary antibody resulting in radioactive precipitate was assessed in replicate for each assay. The mean of nonspecific binding values (NSB) for individual assays was subtracted from each "bound" value. This quantity was divided by free cpm to yield a bound/free (B/F) ratio determined for each standard dilution or rat plasma (serum) dilution: 
$$B/F = \frac{\text{"Bound" (cpm)} - \text{NSB (cpm)}}{\text{"Free" (cpm)}}$$

Standard curves were constructed for each assay system by plotting the replicate values of B/F against the appropriate RP-2 standard dilution (ng/ml diluent). Curves were hand drawn.

##### B. Determination of Rat Plasma or Serum Prolactin Levels

B/F ratios were calculated for dilutions of plasma or serum performed in replicate. Corresponding prolactin levels (ng/ml plasma or serum) were determined graphically. Dilutions yielding values between 1 to 3 ng/ml were used preferentially in subsequent interpretations since the slope of the standard curve was greatest in this region. Final prolactin levels were expressed as a mean of the replicate determinations.

##### C. Evaluation of Interassay Variation

Pooled plasma from six control rats was included in replicate



at two dilutions in each assay system for the longitudinal study. The coefficient of variance was calculated from standard deviation and the mean of pooled plasma prolactin values determined for the longitudinal study assays.

#### D. Serum Osmolalities

The mean serum osmolalities were calculated for the three groups in the third study. The coefficient of variance was previously determined.

#### E. Statistical Analysis

The two tailed student t test for unpaired samples was used to test significant difference between the means of control and experimental groups. Linear regression analysis was performed on the mean prolactin levels in both control and experimental groups in the longitudinal study.





## RESULTS

### I. PLASMA PROLACTIN LEVELS IN POTASSIUM ADAPTATION

#### A. Pilot Study

The mean plasma prolactin level was 78% ( $P < 0.01$ ) lower in the experimental group ( $1.80 \pm 0.36$  ng/ml plasma) compared to the control group ( $8.25 \pm 1.79$  ng/ml plasma, mean  $\pm$  SEM) after twenty-one days of an oral potassium load of 15 to 20 meq  $K^+$ /day (Table 1). There was no apparent increase in plasma prolactin levels starting with  $C_1$  and advancing to  $C_6$  or  $E_2$  advancing to  $E_6$  corresponding to beginning and end of animal decapitations. Only five mean plasma prolactin values were obtained in the experimental group since insufficient plasma was obtained for  $E_1$ .

#### B. Longitudinal Study

Plasma prolactin levels were determined for a total of forty-seven rats by six separate radioimmunoassays. Interassay variability was assessed by running a pooled plasma sample throughout all assays. Bound/Free ratios for both the 1:2 and 1:4 dilutions of pooled plasma corresponded to the interval in the standard curves used to determine plasma prolactin levels in assay samples. All pooled prolactin levels (both 1:2 and 1:4 dilutions) were within 95% confidence intervals calculated by  $\pm 2$  SD from the mean determined for each dilution group. The coefficient of variance was 16% for both dilutions. Linearity of plasma prolactin levels through dilutions was assessed by dividing the mean of pooled plasma levels for all 1:2 dilutions by the mean for 1:4 dilutions. The resulting quotient of 1.87 approximates the actual



dilution factor of 2. (Table XI)

The mean plasma prolactin concentrations for the experimental groups demonstrated a jigsaw pattern when plotted against time (Table IX and Figure IX). Difference between the experimental means was significant in one instance ( $P < 0.05$ ):  $E_{14}-E_{24}$  ( $3.84 \pm 0.45$  ng/ml) vs.  $E_{25}-E_{30}$  ( $7.15 \pm 1.88$  ng/ml, mean  $\pm$  SEM). Since plasma prolactin levels varied significantly within the groups of experimental animals, the mean plasma prolactin levels of control and experimental animals sacrificed on different days were not compared. Plasma prolactin level for experimental rats  $E_{31}$   $E_{32}$   $E_{34}$   $E_{36}$  ( $1.34 \pm 0.37$  ng/ml) was 79% lower ( $P < 0.01$ ) compared to the control group  $C_{10}-C_{13}$  ( $6.29 \pm 1.07$  ng/ml, mean  $\pm$  SEM) after twenty-one days of oral potassium load of 30 to 40 meq  $K^+$ /day/animal. There was no statistically significant difference between the means calculated for the control ( $7.29 \pm 1.68$  ng/ml) or experimental ( $6.29 \pm 1.07$  ng/ml, mean  $\pm$  SEM) animals in total.

Linear regression analysis of sequential mean plasma prolactin levels vs. day of potassium adaptation demonstrated no statistical difference in the slope assuming the slope of the mean plasma prolactin level in the underlying population vs. the same time intervals was zero. The slope determined by linear regression of the three control mean plasma prolactin levels was within the 95% confidence intervals calculated for the slopes of experimental points starting with day 1, 2, 5 and chronologically including points until reaching the mean plasma prolactin concentration corresponding to day 21 of potassium adaptation. Similarly the slopes determined for the experimental points in this manner were within the 95% confidence limits for the slope of



the control mean prolactin levels although this interval of confidence was extremely large since the number of control groups was only three. (Table X, Figure IX).

### C. Third Study

The mean serum prolactin level in six experimental animals ( $4.95 \pm 0.63$  ng/ml) was not significantly different from either control group at day one ( $6.11 \pm 1.74$  ng/ml) or control group at day six ( $5.71 \pm 140$  ng/ml, mean  $\pm$  SEM) of oral potassium load of 30 to 40 meq/day (Table XIII). Furthermore, there was no significant difference between mean plasma or serum prolactin levels determined for: 1. the control groups at day one in the longitudinal study and this study; 2. the control group in the longitudinal study and the experimental group in this study at day five; 3. or the experimental groups in the longitudinal study and this study at day five of potassium adaptation (Tables II, IV, XI). Although the pooled plasma sample was not included in the third study, it appears that mean serum prolactin in this study was not significantly different from mean plasma prolactin levels in the longitudinal study at identical intervals of potassium adaptation. Therefore, no rise in circulating prolactin levels was observed at this time of sacrifice as previously reported in circadian variation of circulating prolactin in the rat.<sup>105,106,107</sup>



## II. SERUM OSMOLALITIES

Serum osmolality was significantly different between the experimental group ( $291.8 \pm 1.80$ ) after five days of oral potassium load and the control group at the start of the study ( $284.2 \pm 0.91$  mosm/kg, mean  $\pm$  SEM;  $P < 0.01$ ). However, there was no significant difference between the experimental and control groups at day five. (Table XII) Plasma potassium levels in rats adapted to a similar potassium load are not significantly different from control rats in previous studies.<sup>79</sup> Hence, it is unlikely that the change in serum osmolality is due to a rise in serum potassium in the present study.





## PILOT STUDY

## Plasma Prolactin Levels in Potassium Adapted Rats by RIA

C<sub>1</sub>-C<sub>6</sub> control rats.E<sub>1</sub>-E<sub>6</sub> potassium adapted rats.Plasma Prolactin  
(ng/ml diluted plasma)mean x  
dilution factor  
ng/ml plasma

## Bound/Free Ratio

sample (dilution)

ng/ml

12.5

1.25

ND

ND

ND

ND

ND

ND

ND

ND

ND

ND

ND

ND

ND

ND

ND

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C<sub>1</sub> (1:1)C<sub>1</sub> (1:2)C<sub>2</sub> (1:2)C<sub>3</sub> (1:2)C<sub>4</sub> (1:2)C<sub>5</sub> (1:2)C<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)E<sub>2</sub> (1:2)E<sub>3</sub> (1:2)E<sub>4</sub> (1:2)E<sub>5</sub> (1:2)E<sub>6</sub> (1:2)

E&lt;



TABLE II

LONGITUDINAL STUDY  
Plasma Prolactin Levels in Control Rats by RIA  
Day 1 of Potassium Adaptation

| Sample(Dilution)                 | Bound/Free Ratio |       |       | Plasma Prolactin)<br>(ng/ml diluted plasma) |       |      | mean<br>ng/ml | mean x<br>dilution factor<br>ng/ml plasma |
|----------------------------------|------------------|-------|-------|---------------------------------------------|-------|------|---------------|-------------------------------------------|
|                                  | 1                | 2     | 3     | 1                                           | 2     | 3    |               |                                           |
| C1 (1:8)                         | 0.847            | 0.832 | 0.762 | 2.45                                        | 2.62  | 3.31 | 2.79          | 22.35                                     |
| C2 (1:2)                         | 0.819            | 0.937 | 0.904 | 2.73                                        | 1.65  | 1.95 | 2.11          | 4.22                                      |
| C3 (1:2)                         | 0.980            | 0.845 | 0.986 | 1.30                                        | 2.50* | 1.25 | 1.28          | 2.55                                      |
| C4 (1:4)                         | 0.732            | 0.745 | 0.752 | 1.05                                        | 0.90  | 0.89 | 0.95          | 3.79                                      |
| C5 (1:2)                         | 0.743            | 0.769 | 0.779 | 0.96                                        | 0.78  | 0.67 | 0.80          | 1.61                                      |
| C6 (1:2)                         | 0.750            | 0.700 | 0.672 | 0.92                                        | 1.27  | 1.50 | 1.23          | 2.46                                      |
| pooled plasma <sup>1</sup> (1:2) | 0.905            | 0.813 | ND    | 1.95                                        | 2.80  | ND   | 2.38          | 4.75                                      |
| pooled plasma <sup>1</sup> (1:4) | 1.000            | 0.951 | 0.938 | 1.15                                        | 1.55  | 1.64 | 1.45          | 5.79                                      |
| pooled plasma <sup>2</sup> (1:4) | 0.775            | 0.717 | ND    | 0.75                                        | 1.14  | ND   | 0.95          | 3.80                                      |
| pooled plasma <sup>2</sup> (1:2) | 0.537            | 0.579 | ND    | 2.80                                        | 2.32  | ND   | 2.56          | 5.12                                      |

mean  $\pm$  SEMC1-C6 6.16  $\pm$  3.26

1. pooled plasma<sup>1</sup>: conversion from B/F to plasma prolactin by Figure III.
2. pooled plasma<sup>2</sup>: conversion from B/F to plasma prolactin by Figure IV.
3. NSB < 10% cpm final precipitate in reference.
4. C1-C3: conversion from B/F to plasma prolactin by Figure III.
5. C4-C6: conversion from B/F to plasma prolactin by Figure IV.



TABLE III

LONGITUDINAL STUDY  
Plasma Prolactin Levels in Experimental Rats by RIA  
Day 3 of Potassium Adaptation

| Sample (Dilution)   | Bound/Free Ratio |       |       | Plasma Prolactin<br>(ng/ml diluted plasma) |      |       | mean<br>ng/ml | mean x<br>dilution factor<br>ng/ml plasma |
|---------------------|------------------|-------|-------|--------------------------------------------|------|-------|---------------|-------------------------------------------|
|                     | 1                | 2     | 3     | 1                                          | 2    | 3     |               |                                           |
| E1 (1:8)            | 0.996            | 1.018 | 0.983 | 1.18                                       | 1.00 | 1.30  | 1.16          | 9.28                                      |
| E2 (1:2)            | 0.784            | 0.754 | 0.742 | 3.08                                       | 3.41 | 3.55  | 3.35          | 6.69                                      |
| E3 (1:8)            | 0.715            | 0.721 | 0.669 | 3.85                                       | 3.78 | 4.50  | 4.04          | 32.34                                     |
| E4 (1:2)            | 0.860            | 0.856 | 0.909 | 2.35                                       | 2.40 | 1.90  | 2.22          | 4.43                                      |
| E5 (1:4)            | 1.034            | 1.032 | 1.092 | 0.87                                       | 0.89 | 0.47* | 0.88          | 3.52                                      |
| E6 (1:2)            | 1.071            | 0.957 | 0.955 | 0.62*                                      | 1.50 | 1.52  | 1.20          | 2.39                                      |
| pooled plasma (1:2) | 0.905            | 0.813 | ND    | 1.95                                       | 2.80 | ND    | 2.38          | 4.75                                      |
| pooled plasma (1:4) | 1.000            | 0.951 | 0.938 | 1.15                                       | 1.55 | 1.64  | 1.45          | 5.79                                      |

mean  $\pm$  SEME1-E3      9.78  $\pm$  4.62

1. B/F ratio to plasma prolactin by Figure III including pooled plasma.
2. NSB < 10% cpm final precipitate in reference.
3. \*value excluded due to large variation.





LONGITUDINAL STUDY  
Plasma Prolactin Levels in Experimental Rats by RIA

| Sample (Dilution) | Bound/Free Ratio |       |       | Plasma Prolactin<br>(ng/ml diluted plasma) |      |      | mean<br>ng/ml | mean x<br>dilution factor<br>ng/ml plasma |
|-------------------|------------------|-------|-------|--------------------------------------------|------|------|---------------|-------------------------------------------|
|                   | 1                | 2     | 3     | 1                                          | 2    | 3    |               |                                           |
| E7 (1:2)          | 0.791            | 0.748 | 0.746 | 0.65                                       | 0.92 | 0.95 | 0.84          | 1.68                                      |
| E8 (1:2)          | 0.748            | 0.775 | 0.737 | 0.92                                       | 0.75 | 1.00 | 0.89          | 1.78                                      |
| E9 (1:4)          | 0.570            | 0.532 | 0.574 | 2.42                                       | 2.90 | 2.37 | 2.56          | 10.24                                     |
| E10 (1:4)         | 0.771            | 0.745 | 0.750 | 0.77                                       | 0.96 | 0.91 | 0.88          | 3.52                                      |
| E11 (1:4)         | 0.594            | 0.595 | 0.548 | 2.16                                       | 2.15 | 2.66 | 2.32          | 9.28                                      |
| E12 (1:2)         | 0.783            | 0.782 | 0.773 | 0.70                                       | 0.72 | 0.81 | 0.74          | 1.48                                      |
| pooled plasma     | 0.537            | 0.579 | ND    | 2.80                                       | 2.32 | ND   | 2.56          | 5.12                                      |
| pooled plasma     | 0.775            | 0.717 | ND    | 0.75                                       | 1.14 | ND   | 0.95          | 3.80                                      |

mean  $\pm$  SEM

E7-E12 4.66  $\pm$  1.64

1. Conversion of B/F ratio to plasma prolactin by Figure IV including pooled plasma.
2. NSB < 10% cpm final precipitate in reference



LONGITUDINAL STUDY  
Plasma Prolactin Levels in Control and Experimental Rats  
Day 9 Potassium Adaptation

| Sample (dilution) | Bound/Free |       |       | Plasma Prolactin<br>(ng/ml diluted plasma) |      |      | mean<br>ng/ml | mean x<br>dilution factor<br>ng/ml plasma |
|-------------------|------------|-------|-------|--------------------------------------------|------|------|---------------|-------------------------------------------|
|                   | 1          | 2     | 3     | 1                                          | 2    | 3    |               |                                           |
| C7 (1:2)          | 0.643      | 0.613 | 0.657 | 1.50                                       | 1.83 | 1.34 | 1.56          | 3.11                                      |
| C8 (1:4)          | 0.681      | 0.608 | 0.629 | 1.10                                       | 1.90 | 1.67 | 1.56          | 6.23                                      |
| C9 (1:2)          | 0.600      | 0.570 | 0.560 | 2.00                                       | 2.37 | 2.53 | 2.30          | 4.60                                      |
| E13 (1:4)         | 0.629      | 0.573 | 0.599 | 1.66                                       | 2.33 | 2.00 | 2.00          | 7.99                                      |
| E14 (1:2)         | 0.573      | 0.657 | 0.620 | 2.35                                       | 1.35 | 1.78 | 1.83          | 3.65                                      |
| E15 (1:8)         | 0.641      | 0.586 | 0.573 | 1.53                                       | 2.14 | 2.34 | 2.00          | 16.02                                     |
| E16 (1:4)         | 0.630      | 0.669 | 0.635 | 1.64                                       | 1.23 | 1.60 | 1.49          | 5.96                                      |
| E17 (1:4)         | 0.610      | 0.605 | 0.611 | 1.88                                       | 1.95 | 1.87 | 1.90          | 7.60                                      |
| E18 (1:8)         | 0.616      | 0.607 | 0.605 | 1.81                                       | 1.93 | 1.95 | 1.90          | 15.17                                     |
| pooled plasma     | 0.566      | 0.565 | 0.523 | 2.45                                       | 2.46 | 3.17 | 2.69          | 5.39                                      |
| pooled plasma     | 0.673      | 0.657 | 0.660 | 1.18                                       | 1.34 | 1.31 | 1.28          | 5.11                                      |

C7-C9 4.65 ± 0.90 t = -1.546 P > 0.10  
E13-E18 9.40 ± 2.06

1. Conversion B/F ratio to plasma prolactin values by Figure V.
2. NSB < 10% cpm final precipitate in reference.



Plasma Prolactin Levels in Experimental Rats by RIA  
Day 12 of Potassium Adaptation

| Sample(Dilution)    | Bound/Free Ratio |       |       | Plasma Prolactin Level<br>(ng/ml diluted plasma) |      |      | mean<br>ng/ml | mean x<br>dilution<br>factor<br>ng/ml plasma |
|---------------------|------------------|-------|-------|--------------------------------------------------|------|------|---------------|----------------------------------------------|
|                     | 1                | 2     | 3     | 1                                                | 2    | 3    |               |                                              |
| E19 (1:2)           | 0.810            | 0.828 | 0.900 | 1.07                                             | 0.97 | 0.58 | 0.87          | 1.75                                         |
| E20 (1:2)           | 0.869            | 0.833 | 0.806 | 0.75                                             | 0.98 | 1.10 | 0.94          | 1.89                                         |
| E21 (1:8)           | 0.821            | 0.866 | 0.853 | 1.03                                             | 0.77 | 0.85 | 0.88          | 7.07                                         |
| E22 (1:2)           | 0.751            | 0.754 | 0.730 | 1.43                                             | 1.42 | 1.57 | 1.47          | 2.95                                         |
| E23 (1:4)           | 0.734            | 0.712 | 0.720 | 1.55                                             | 1.68 | 1.62 | 1.62          | 6.47                                         |
| E24 (1:2)           | 0.755            | 0.742 | 0.758 | 1.42                                             | 1.50 | 1.40 | 1.44          | 2.88                                         |
| pooled plasma (1:2) | 0.700            | 7.48  | 7.26  | 1.75                                             | 1.45 | 1.60 | 1.60          | 3.20                                         |

mean  $\pm$  SEM

E19-E24 3.84  $\pm$  0.95

1. Conversion of B/F ratio to plasma prolactin levels by Figure VI including pooled plasma.
2. NSB <10% cpm final precipitate in reference.



# LONGITUDINAL STUDY

## Plasma Prolactin Levels in Experimental Rats by RIA

Day 17 of Potassium Adaptation

| Sample (Dilution) | Bound/Free Ratio |       |       | Plasma Prolactin<br>(ng/ml diluted plasma) |      |      | mean<br>ng/ml | mean x<br>dilution factor<br>ng/ml plasma |
|-------------------|------------------|-------|-------|--------------------------------------------|------|------|---------------|-------------------------------------------|
|                   | 1                | 2     | 3     | 1                                          | 2    | 3    |               |                                           |
| E25 (1:4)         | 0.727            | 0.798 | 0.719 | 1.50                                       | 1.36 | 1.55 | 1.47          | 5.88                                      |
| E26 (1:4)         | 0.701            | 0.731 | 0.736 | 1.61                                       | 1.48 | 1.45 | 1.51          | 6.05                                      |
| E27 (1:4)         | 0.670            | 0.641 | 0.675 | 1.90                                       | 2.13 | 1.85 | 1.63          | 6.51                                      |
| E28 (1:4)         | 0.822            | 0.806 | 0.798 | 0.93                                       | 1.01 | 1.05 | 1.00          | 3.99                                      |
| E29 (1:8)         | 0.602            | 0.661 | 0.743 | 2.45                                       | 1.96 | 1.40 | 4.08          | 16.33                                     |
| E30 (1:4)         | 0.566            | 0.675 | 0.710 | 1.45                                       | 0.97 | 1.00 | 1.04          | 4.16                                      |
| pooled plasma     | 0.615            | 0.602 | 0.608 | 2.33                                       | 2.45 | 2.38 | 2.39          | 4.77                                      |
| pooled plasma     | 0.762            | 0.774 | ND    | 1.55                                       | 1.20 | ND   | 1.38          | 5.50                                      |

mean  $\pm$  SEM  
E25-E30 7.15  $\pm$  1.88

1. Conversion of B/F ratios to plasma prolactin levels by Figure VII.
2. NSB <10% cpm precipitate in reference.
3. ND = not done





# LONGITUDINAL STUDY

## Plasma Prolactin Levels in Control and Experimental Rats by RIA

### Day 22 of Potassium Adaptation

| Sample (Dilution)                | Bound/Free Ratio |       |       | Plasma Prolactin<br>(ng/ml diluted plasma) |       |      | mean<br>ng/ml | mean x<br>dilution factor<br>ng/ml plasma |
|----------------------------------|------------------|-------|-------|--------------------------------------------|-------|------|---------------|-------------------------------------------|
|                                  | 1                | 2     | 3     | 1                                          | 2     | 3    |               |                                           |
| C10 (1:4)                        | 0.733            | 0.725 | 0.723 | 1.48                                       | 1.50  | 1.52 | 1.50          | 6.00                                      |
| C11 (1:8)                        | 0.713            | 0.668 | 0.708 | 1.60                                       | 1.92  | 1.63 | 1.72          | 13.73                                     |
| C12 (1:8)                        | 0.703            | 0.749 | 0.730 | 1.65                                       | 1.37  | 1.48 | 1.50          | 12.00                                     |
| C13 (1:8)                        | 0.738            | 0.711 | 0.721 | 1.45                                       | 1.60  | 1.53 | 1.53          | 12.21                                     |
| E31 (1:2)                        | 1.026            | 0.936 | 0.952 | <0.25                                      | 0.42  | 0.34 | 0.38          | 0.76                                      |
| E32 (1:2)                        | 0.939            | 0.999 | 0.975 | 0.39                                       | <0.25 | 0.25 | 0.32          | 0.64                                      |
| E34 (1:2)                        | 0.781            | 0.831 | 0.824 | 1.15                                       | 0.94  | 0.96 | 1.02          | 2.04                                      |
| E36 (1:2)                        | 0.803            | 0.826 | 0.849 | 1.07                                       | 0.95  | 0.84 | 0.95          | 1.91                                      |
| pooled plasma <sup>1</sup> (1:2) | 0.615            | 0.602 | 0.608 | 2.33                                       | 2.45  | 2.38 | 2.33          | 4.77                                      |
| pooled plasma <sup>1</sup> (1:4) | 0.762            | 0.774 | ND    | 1.55                                       | 1.20  | ND   | 1.25          | 5.50                                      |
| pooled plasma <sup>2</sup> (1:2) | 0.625            | 0.570 | 0.568 | 2.13                                       | 2.52  | 2.54 | 2.40          | 4.79                                      |
| pooled plasma <sup>2</sup> (1:4) | 0.780            | 0.792 | ND    | 1.20                                       | 1.14  | ND   | 1.17          | 4.68                                      |

| mean ± SEM              |                             | t     | P     |
|-------------------------|-----------------------------|-------|-------|
| C10-C13<br>E31E32E34E36 | 10.98 ± 1.71<br>1.34 ± 0.37 | 5.527 | <0.01 |

1. C<sub>10</sub> C<sub>11</sub> pooled plasma<sup>1</sup> B/F ratios converted to plasma prolactin levels by Figure VII.
2. C<sub>12</sub> C<sub>13</sub> pooled plasma<sup>2</sup> B/F ratios converted to plasma prolactin levels by Figure VIII.
3. NSB <10% precipitate in reference for standard curves VII, VIII.
4. Rat numbers 33,35 died one day prior to sacrifice.



## LONGITUDINAL STUDY

## SUMMARY OF PLASMA PROLACTIN LEVELS

| <u>Sample Numbers</u> | plasma prolactin ng/ml<br>mean $\pm$ SEM |
|-----------------------|------------------------------------------|
| C1-C6                 | 6.16 $\pm$ 3.26                          |
| C7-C9                 | 4.65 $\pm$ 0.90                          |
| C10-C13               | 10.98 $\pm$ 1.71                         |
| C1-C13                | 7.29 $\pm$ 1.68                          |
| E1-E6                 | 9.78 $\pm$ 4.62                          |
| E7-E12                | 4.66 $\pm$ 1.64                          |
| E13-E18               | 9.40 $\pm$ 2.06                          |
| E19-E24               | 3.84 $\pm$ 0.95                          |
| E25-E30               | 7.15 $\pm$ 1.88                          |
| E31, 32, 34, 36       | 1.34 $\pm$ 0.37                          |
| E1-E36                | 6.29 $\pm$ 1.07                          |

1. Statistical analysis between groups sacrificed on same day

C1-C9 vs. E13-E18 P >0.10

C10-C13 vs. E31, 32, 34, 36 P <0.01

2. Statistical analysis between experimental groups

E19-E24 vs. E13-E18 P <0.05

3. No significant difference between C1-C13 and E1-E56



TABLE X

## LONGITUDINAL STUDY

Regression Analysis of Mean Plasma Prolactin (y) vs. Day of Potassium Adaptation(x)

| Points in Regression Analysis<br>Day # | Sy.x             | intercept $\pm$ SE | slope $\pm$ SE     | slope     |       | 95% confidence<br>upper | slope<br>confidence<br>lower | intervals<br>lower |
|----------------------------------------|------------------|--------------------|--------------------|-----------|-------|-------------------------|------------------------------|--------------------|
|                                        |                  |                    |                    | $t_{n-2}$ | P     |                         |                              |                    |
| Control                                | 1,2,5            | 4.55 $\pm$ 2.48    | 0.25 $\pm$ 0.18    | 1.403     | >0.10 | 23.25                   |                              | -22.74             |
| Experimental                           | 1,2,5            | 7.99 $\pm$ 4.31    | (-)0.37 $\pm$ 1.26 | -0.297    | >0.10 | 3.63                    |                              | - 4.38             |
|                                        | 1,2,5,9          | 7.90 $\pm$ 2.04    | 0.24 $\pm$ 2.89    | 0.083     | >0.10 | 12.65                   |                              | -12.17             |
|                                        | 1,2,5,9,12       | 7.90 $\pm$ 2.04    | (-)0.34 $\pm$ 0.30 | -1.13     | >0.10 | 2.84                    |                              | - 3.52             |
|                                        | 1,2,5,9,12,17    | 7.26 $\pm$ 1.65    | (-)0.05 $\pm$ 0.17 | -0.29     | >0.10 | 2.73                    |                              | - 2.83             |
|                                        | 1,2,5,9,12,17,22 | 8.19 $\pm$ 1.80    | (-)0.22 $\pm$ 0.15 | 1.47      | >0.10 | 2.35                    |                              | - 2.79             |

1. In all instances, slope described by combinations of experimental points fall within 1 unit of standard error of the slope described by control points
2. Slope described by control points falls within 95% confidence intervals described by all experimental points (confidence interval = slope  $\pm$   $t_{n-2,0.05}$  [SE slope]).
3. Calculations for P value based on two tailed student T test with population slope = 0
4. See figure IX.



TABLE XI

## INTERASSAY VARIATION IN LONGITUDINAL STUDY

Plasma Prolactin Levels of Pooled Plasma  
(ng/ml plasma)

| Standard Curve<br>(Figure #) | B/F ratio |       |       | Plasma Prolactin<br>(ng/ml diluted plasma) |      |      | mean<br>ng/ml | mean<br>x dilution factor<br>ng/ml plasma |
|------------------------------|-----------|-------|-------|--------------------------------------------|------|------|---------------|-------------------------------------------|
|                              | 1         | 2     | 3     | 1                                          | 2    | 3    |               |                                           |
| II (1:2)                     | 0.905     | 0.813 | ND    | 1.95                                       | 2.80 | ND   | 2.38          | 4.75                                      |
| II (1:4)                     | 1.000     | 0.951 | 0.938 | 1.15                                       | 1.55 | 1.64 | 1.45          | 5.79                                      |
| III (1:2)                    | 0.537     | 0.579 | ND    | 2.80                                       | 2.32 | ND   | 2.56          | 5.12                                      |
| IV (1:2)                     | 0.566     | 0.565 | 0.523 | 2.45                                       | 2.46 | 3.17 | 2.69          | 5.39                                      |
| IV (1:4)                     | 0.673     | 0.657 | 0.660 | 1.18                                       | 1.34 | 1.31 | 1.28          | 5.11                                      |
| V (1:2)                      | 0.700     | 0.748 | 0.726 | 1.75                                       | 1.45 | 1.60 | 1.60          | 3.20                                      |
| VI (1:2)                     | 0.615     | 0.602 | 0.602 | 2.33                                       | 2.45 | 2.38 | 2.39          | 4.77                                      |
| VI (1:4)                     | 0.762     | 0.774 | ND    | 1.55                                       | 1.20 | ND   | 1.38          | 5.50                                      |
| VII (1:2)                    | 0.625     | 0.570 | 0.568 | 2.13                                       | 2.52 | 2.54 | 2.40          | 4.79                                      |
| VII (1:4)                    | 0.780     | 0.792 | ND    | 1.20                                       | 1.14 | ND   | 1.17          | 4.68                                      |

95% confidence limits ng/ml plasma  
( $\pm 2$  SD units)upper  
lower3.10  
1.58CV  
16%SEM  
0.16SD  
0.38mean  
(ng/ml plasma)

2.34

1:2 prior to conversion by dilution factor

1:4 prior to conversion by dilution factor

1:2 after conversion by dilution factor

1:4 after conversion by dilution factor

1.65

1.65

3.15

6.19

3.42

6.54

1. All pooled plasma prolactin levels lie within 95% confidence intervals for the respective dilutions.

2. Indication of dilutional linearity:  $\frac{\text{mean (1:2)}}{\text{mean (1:4)}} = \frac{2.34}{1.25} = 1.87$

3. CV = coefficient of variance:  $\frac{\text{SD}}{\text{mean}} \times 100$





THIRD STUDY  
Serum Osmolalities

C1-C6      before start of potassium adaptation (control group)  
C7-C13    day 5 potassium adaptation (control group)  
E1-E6      day 5 potassium adaptation (experimental group)

| sample | mosm/kg serum | sample | mosm/kg serum | sample | mosm/kg serum |
|--------|---------------|--------|---------------|--------|---------------|
| C1     | 286           | C7     | 287           | E1     | 299           |
| C2     | 286           | C8     | 302           | E2     | 293           |
| C3     | 280           | C9     | 289           | E3     | 289           |
| C4     | 285           | C10    | 285           | E4     | 291           |
| C5     | 284           | C11    | 289           | E5     | 293           |
| C6     | 284           | C12    | 285           | E6     | 286           |
|        |               | C13    | 285           |        |               |

|        | mean   | SD   | SEM  |
|--------|--------|------|------|
| C1-C6  | 284.17 | 2.23 | 0.91 |
| C7-C13 | 288.86 | 6.07 | 2.29 |
| E1-E6  | 291.83 | 4.40 | 1.80 |

Significant Differences  
(based on two tailed student t test for unmatched sample pairs)

|        |            |       |        |
|--------|------------|-------|--------|
|        |            | t     | p      |
| C1-C6  | vs. C7-C13 | -1.78 | > 0.10 |
| C1-C6  | vs. E1-E6  | -3.81 | < 0.01 |
| C7-C13 | vs. E1-E6  | -1.00 | > 0.10 |



TABLE XIII

THIRD STUDY  
(all sera collected at 1900 hrs)  
Serum Prolactin Levels in Control and Experimental Rats by RIA  
C<sub>1</sub>-C<sub>6</sub> Day 1 Potassium Adaptation  
C<sub>1</sub>-C<sub>13</sub>, E<sub>1</sub>-E<sub>6</sub> Day 5 Potassium Adaptation

| Sample(Dilution)      | Bound/Free Ratio |       |       | Serum Prolactin<br>(ng/ml diluted serum) |       |      | mean<br>ng/ml | mean x<br>dilution factor<br>ng/ml serum |
|-----------------------|------------------|-------|-------|------------------------------------------|-------|------|---------------|------------------------------------------|
|                       | 1                | 2     | 3     | 1                                        | 2     | 3    |               |                                          |
| C <sub>1</sub> (1:2)  | 0.720            | 0.751 | 0.664 | 1.06                                     | 0.81  | 1.55 | 1.14          | 2.28                                     |
| C <sub>2</sub> (1:4)  | 0.708            | 0.718 | 0.702 | 1.15                                     | 1.07  | 1.22 | 1.15          | 4.58                                     |
| C <sub>3</sub> (1:4)  | 0.577            | 0.547 | 0.559 | 2.44                                     | 2.60  | 2.65 | 2.56          | 10.25                                    |
| C <sub>4</sub> (1:2)  | 0.698            | 0.666 | 0.645 | 1.23                                     | 1.52  | 1.72 | 1.49          | 2.98                                     |
| C <sub>5</sub> (1:4)  | 0.747            | 0.715 | 0.720 | 0.83                                     | 1.10  | 1.05 | 0.99          | 3.97                                     |
| C <sub>6</sub> (1:4)  | 0.505            | 0.503 | 0.539 | 3.30                                     | 3.32  | 2.83 | 3.15          | 12.60                                    |
| C <sub>7</sub> (1:4)  | 0.601            | 0.671 | 0.658 | 2.17                                     | 1.48  | 1.60 | 1.75          | 7.00                                     |
| C <sub>8</sub> (1:4)  | 0.725            | 0.710 | 0.713 | 1.00                                     | 1.14  | 1.11 | 1.08          | 4.33                                     |
| C <sub>9</sub> (1:4)  | 0.526            | 0.550 | 0.529 | 3.04                                     | 2.75  | 3.00 | 2.93          | 11.72                                    |
| C <sub>10</sub> (1:2) | 0.653            | 0.631 | 0.652 | 1.65                                     | 1.81  | 1.66 | 1.71          | 3.41                                     |
| C <sub>11</sub> (1:2) | 0.727            | 0.710 | 0.740 | 1.00                                     | 1.13  | 0.87 | 1.00          | 2.00                                     |
| C <sub>12</sub> (1:2) | 0.688            | 0.730 | 0.699 | 1.32                                     | 0.97  | 1.23 | 1.17          | 2.35                                     |
| C <sub>13</sub> (1:4) | 0.618            | 0.598 | 0.556 | 2.00                                     | 2.20  | 2.68 | 2.29          | 9.17                                     |
| E <sub>1</sub> (1:4)  | 0.632            | 0.645 | 0.654 | 1.85                                     | 1.70  | 1.62 | 1.72          | 6.89                                     |
| E <sub>2</sub> (1:4)  | 0.705            | 0.682 | 0.709 | 1.17                                     | 1.38  | 1.14 | 1.23          | 4.92                                     |
| E <sub>3</sub> (1:4)  | 0.686            | 0.690 | 0.657 | 1.35                                     | 1.30  | 1.60 | 1.42          | 5.67                                     |
| E <sub>4</sub> (1:2)  | 0.604            | 0.598 | 0.590 | 2.15                                     | 2.20  | 2.30 | 2.22          | 4.44                                     |
| E <sub>5</sub> (1:4)  | 0.682            | 0.686 | 0.678 | 1.40                                     | 1.30  | 1.42 | 1.37          | 5.49                                     |
| E <sub>6</sub> (1:2)  | 0.703            | 0.680 | 0.715 | 1.20                                     | 1.90* | 1.10 | 1.15          | 2.30                                     |

## Statistical Analysis

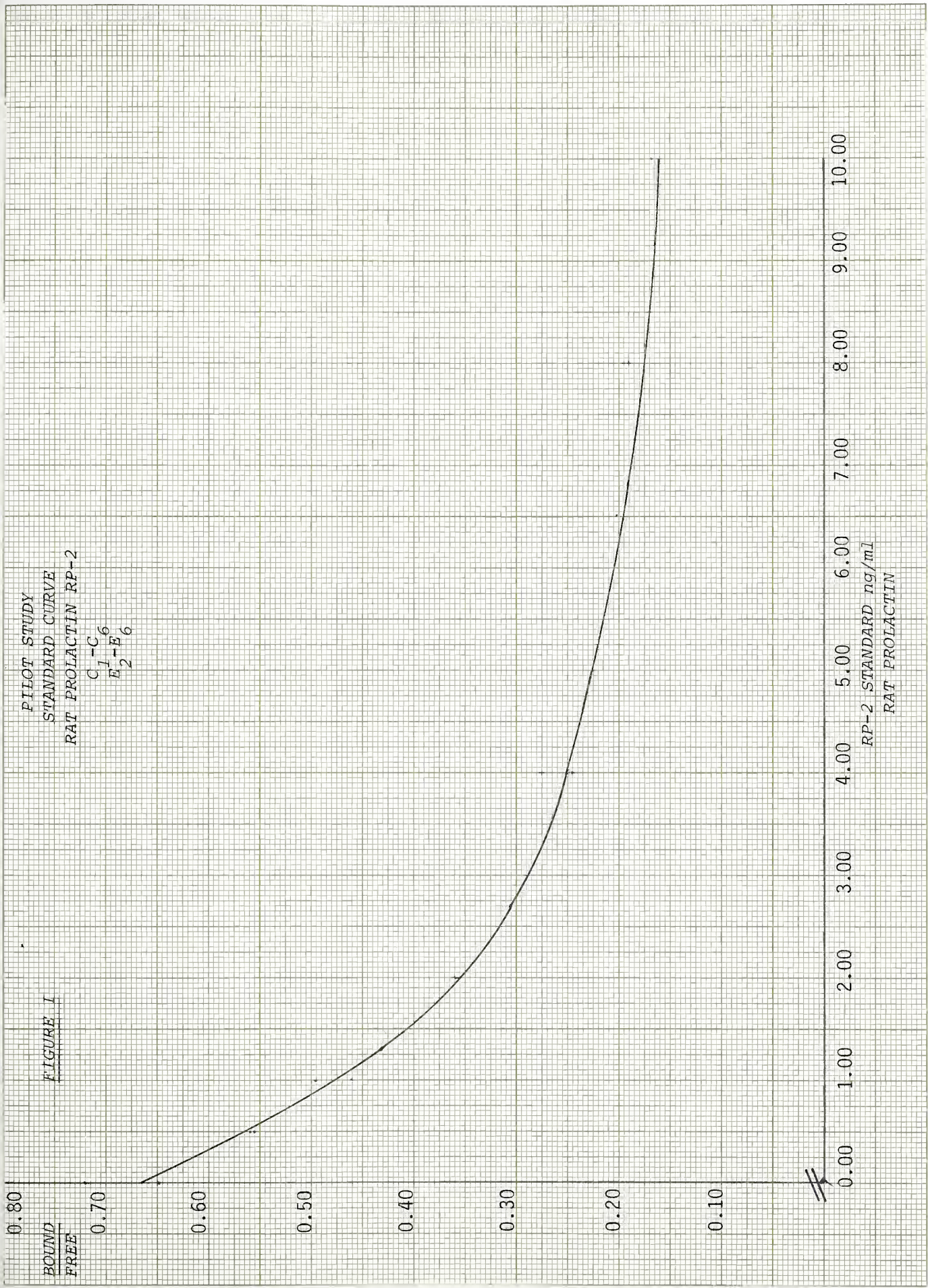
mean  $\pm$  SEM  
ng/ml serum

C<sub>1</sub>-C<sub>6</sub> 6.11  $\pm$  1.74  
C<sub>7</sub>-C<sub>13</sub> 5.71  $\pm$  1.40  
E<sub>1</sub>-E<sub>6</sub> 4.95  $\pm$  0.63

- C<sub>1</sub>-C<sub>6</sub> vs. C<sub>7</sub>-C<sub>13</sub> t=0.181 P > 0.10  
C<sub>1</sub>-C<sub>6</sub> vs. E<sub>1</sub>-E<sub>6</sub> t=0.627 P > 0.10  
C<sub>7</sub>-C<sub>13</sub> vs. E<sub>1</sub>-E<sub>6</sub> t=0.501 P > 0.10
1. Conversion from B/F ratio to serum prolactin levels by figure VIII.  
2. NSB < 10% precipitate in reference.  
3. \*value excluded due to large variation.







PILOT STUDY  
STANDARD CURVE  
RAT PROLACTIN RP-2  
C<sub>1</sub>-C<sub>6</sub>  
E<sub>2</sub>-E<sub>6</sub>

FIGURE 1





FIGURE II

PILOT STUDY  
STANDARD CURVE  
RAT PROLACTIN RP-2  
C<sub>1</sub> 1:10 DILUTION

$\frac{\text{BOUND}}{\text{FREE}}$

0.70

0.60

0.50

0.40

0.30

0.20

0.10

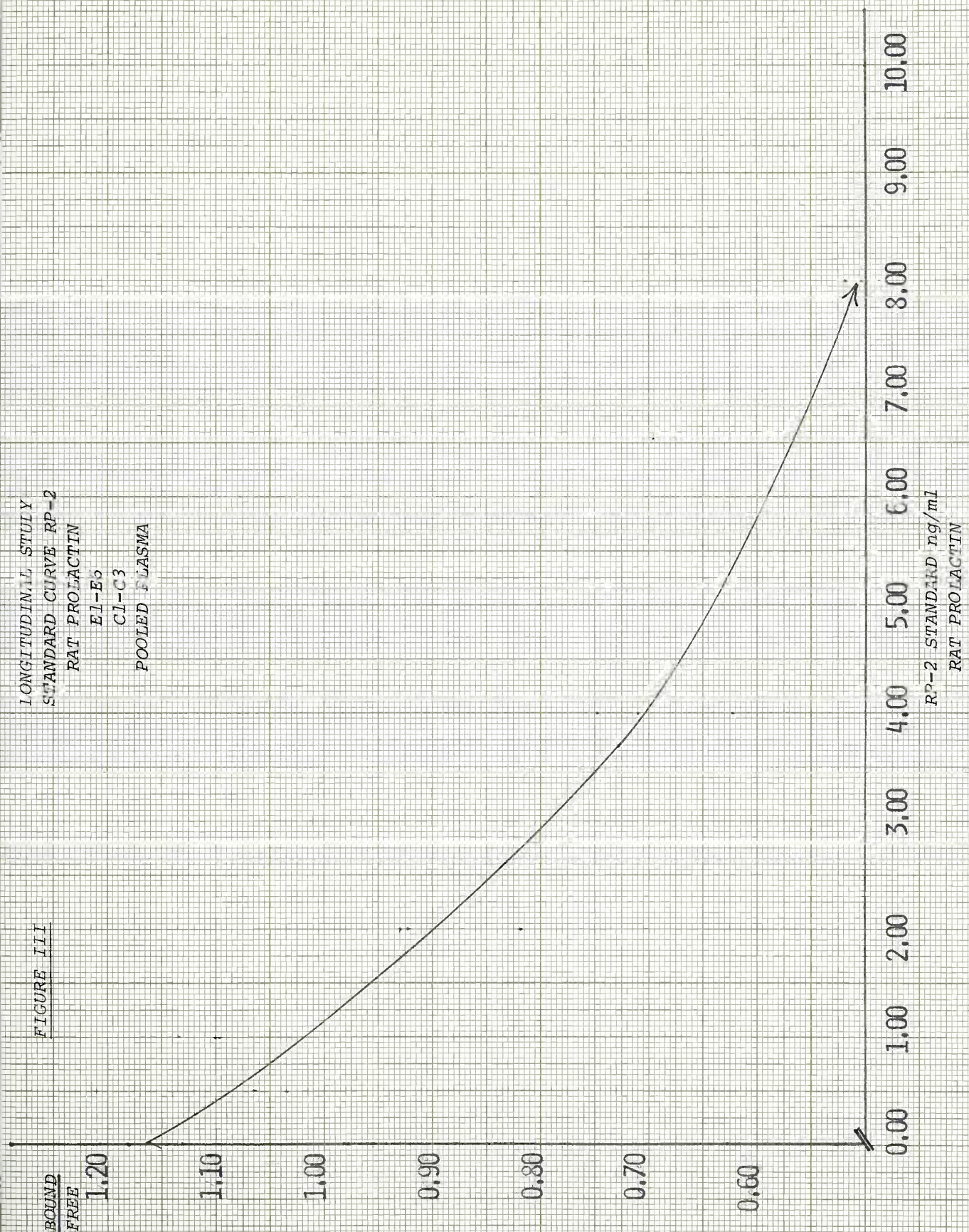
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00

RP-2 STANDARD ng/ml  
RAT PROLACTIN





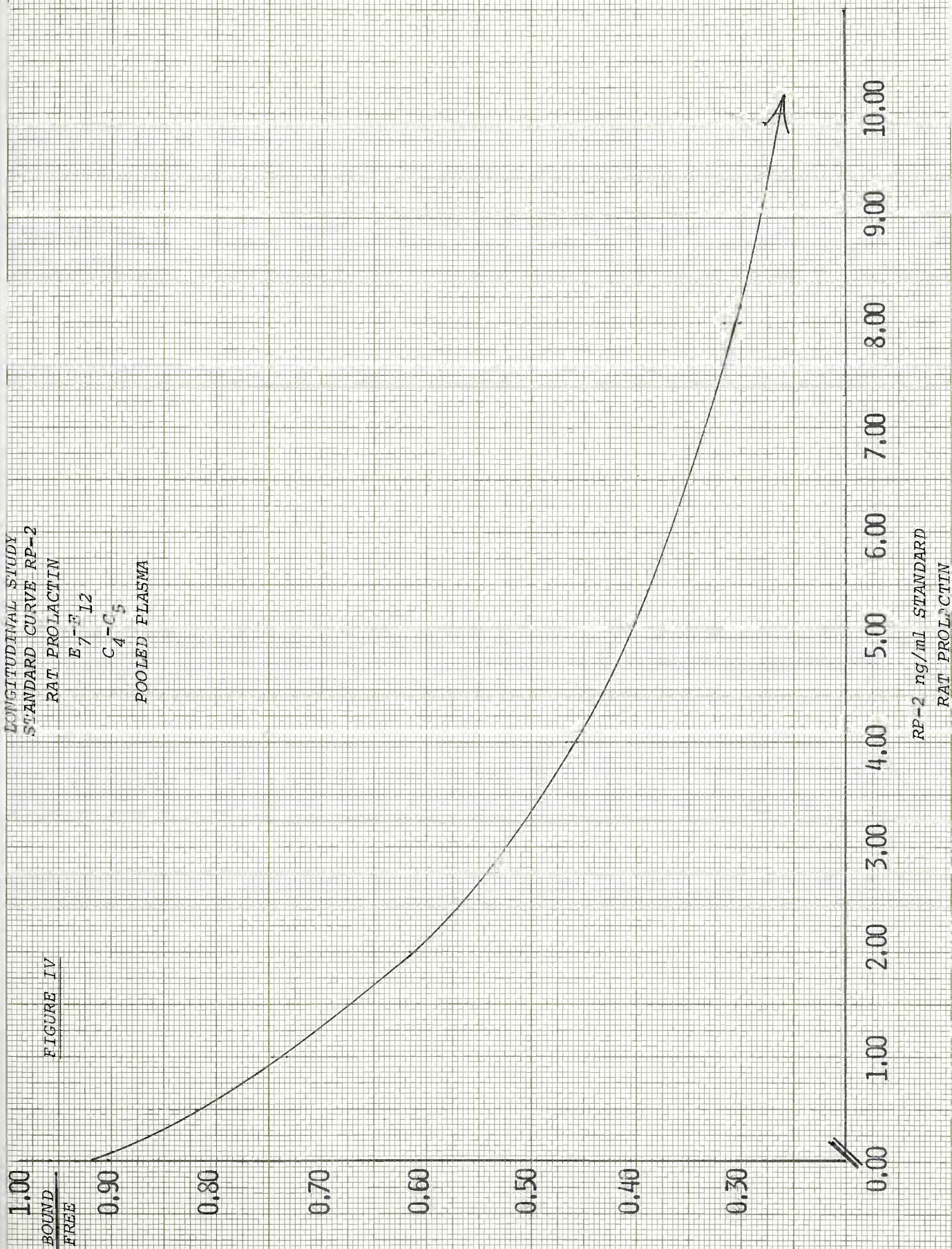
LONGITUDINAL STUDY  
STANDARD CURVE RP-2  
RAT PROLACTIN  
E1-E6  
C1-C3  
POOLED PLASMA







LONGITUDINAL STUDY  
STANDARD CURVE RP-2  
RAT PROLACTIN  
E7-3  
C4-C5  
POOLED PLASMA







LONGITUDINAL STUDY  
STANDARD CURVE RP-2  
RAT PROLACTIN  
E13-E18  
C7-C9  
POOLED PLASMA

FIGURE V

BOUND  
FREE

0.90

0.80

0.70

0.60

0.50

0.40

0.30

0.00

1.00

2.00

3.00

4.00

5.00

6.00

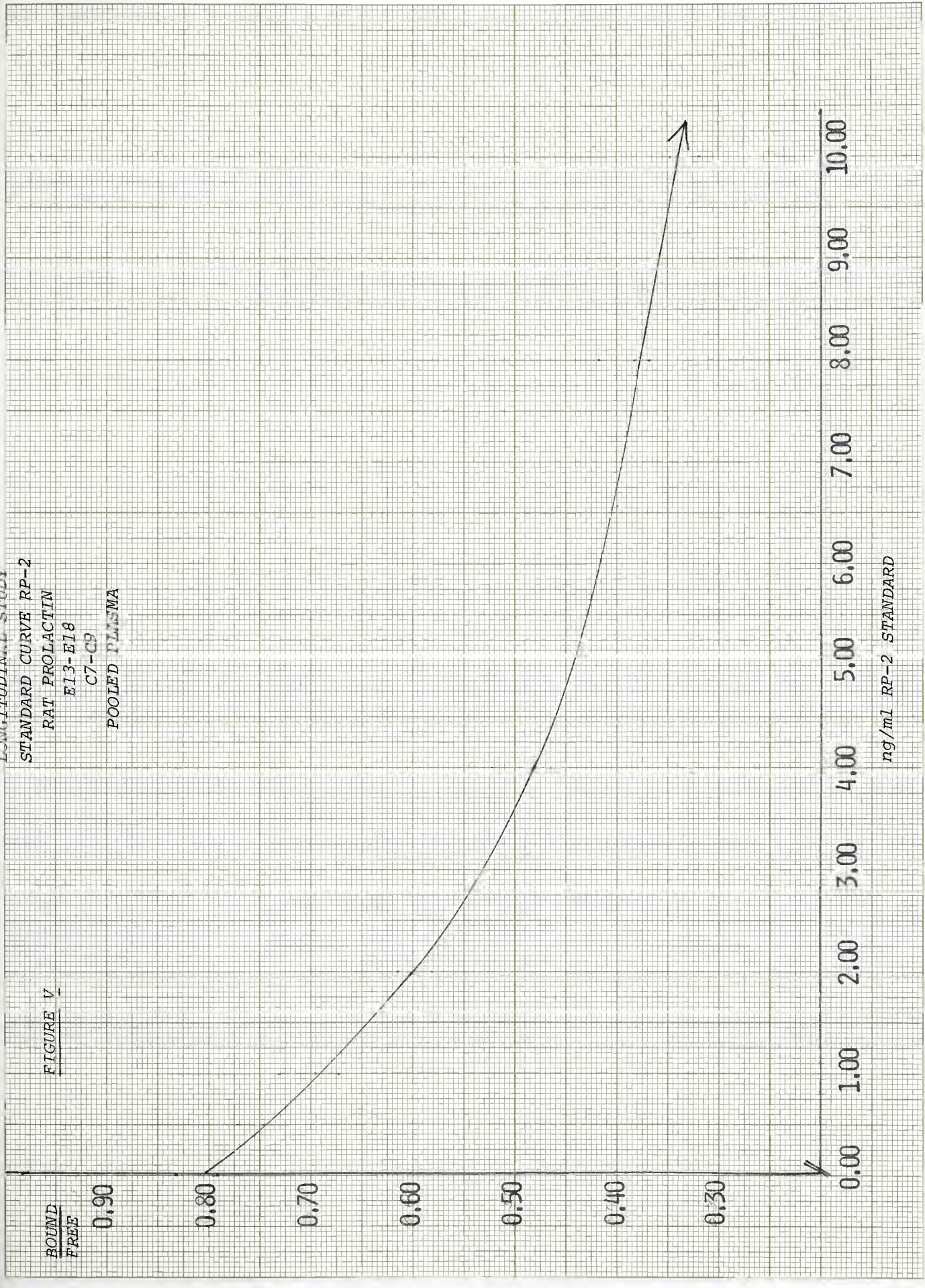
7.00

8.00

9.00

10.00

ng/ml RP-2 STANDARD







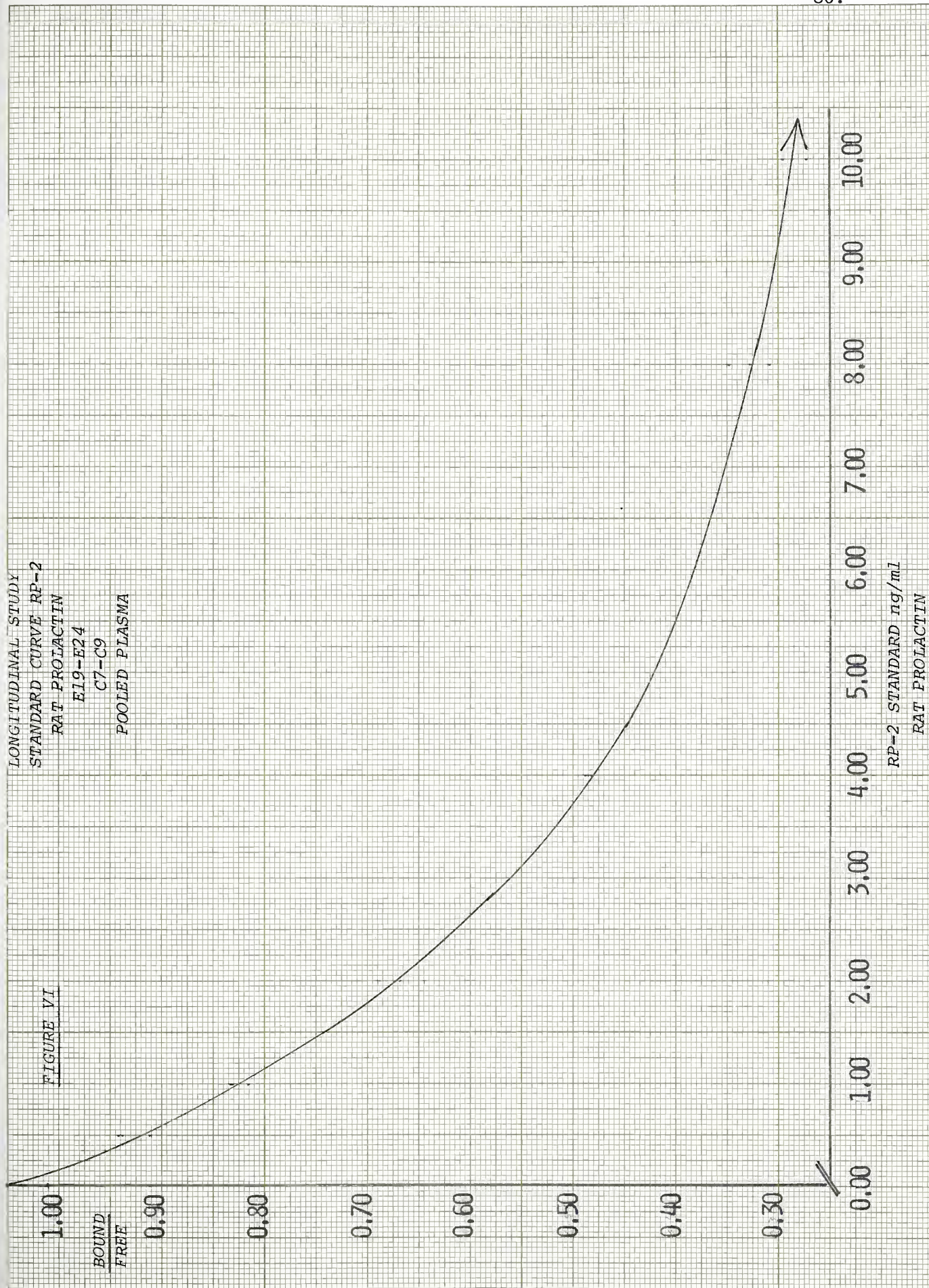


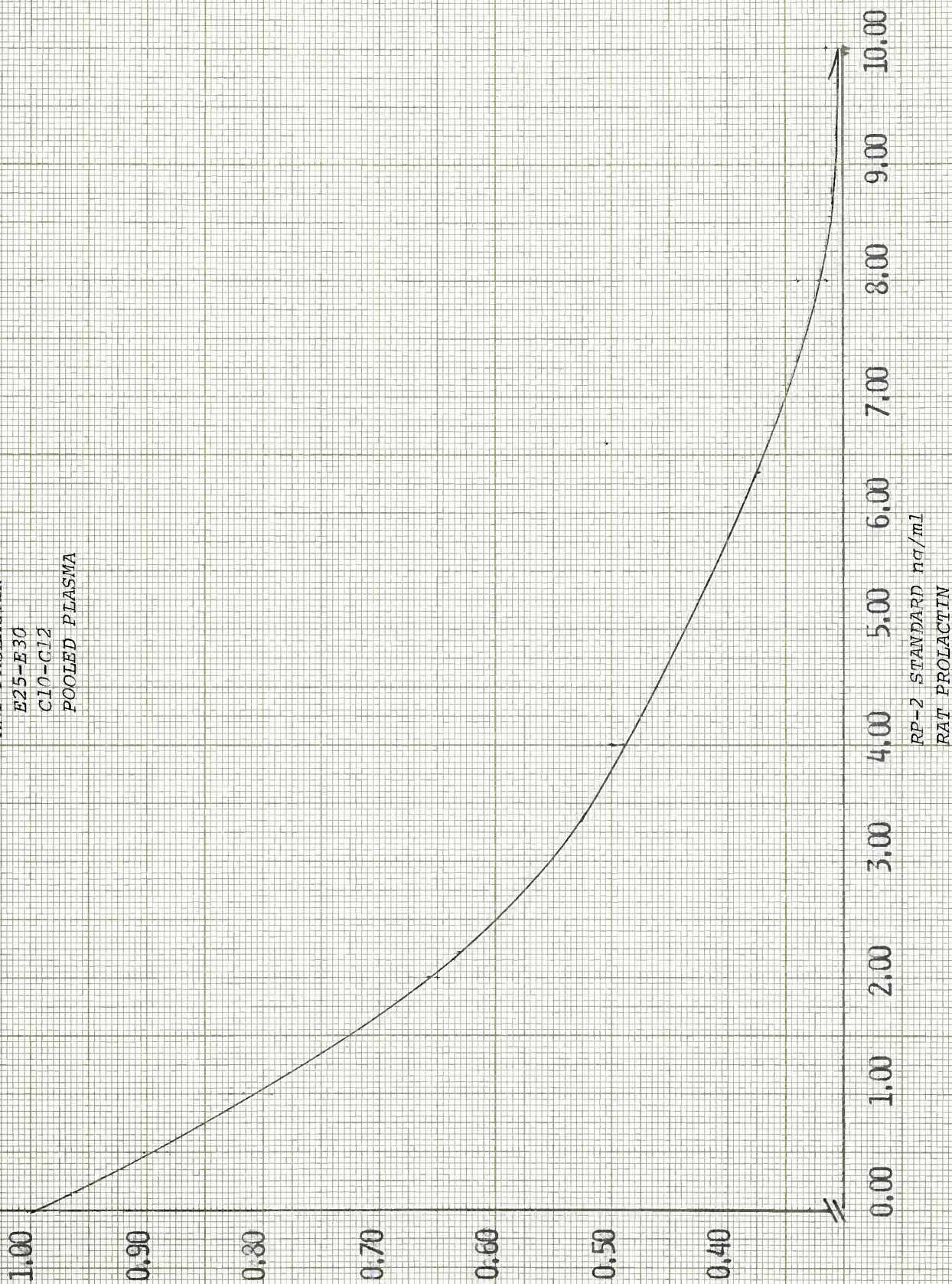




FIGURE VII

LONGITUDINAL STUDY  
STANDARD CURVE  
RAT PROLACTIN  
E25-E30  
C10-C12  
POOLED PLASMA

BOUND  
FREE

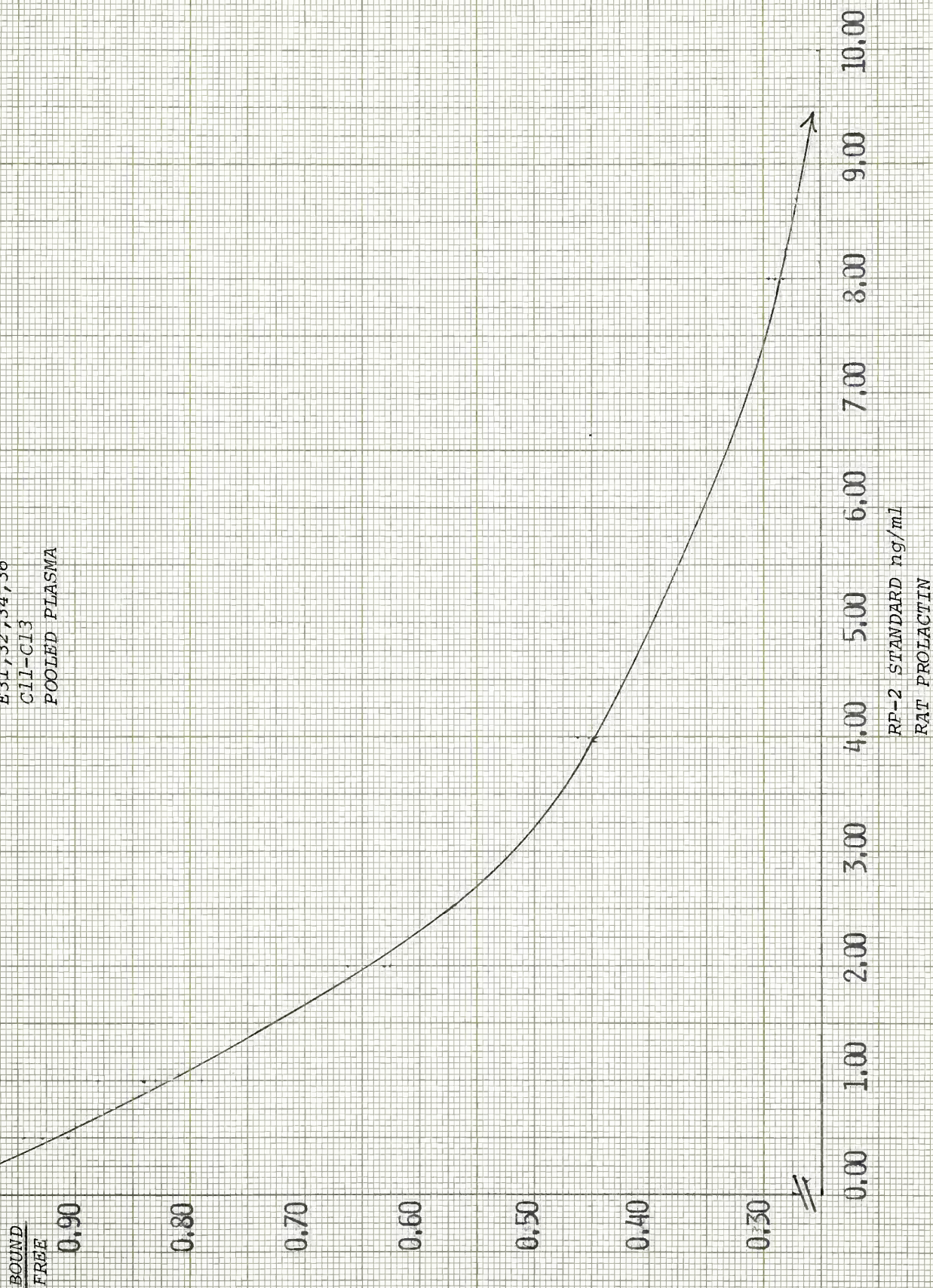






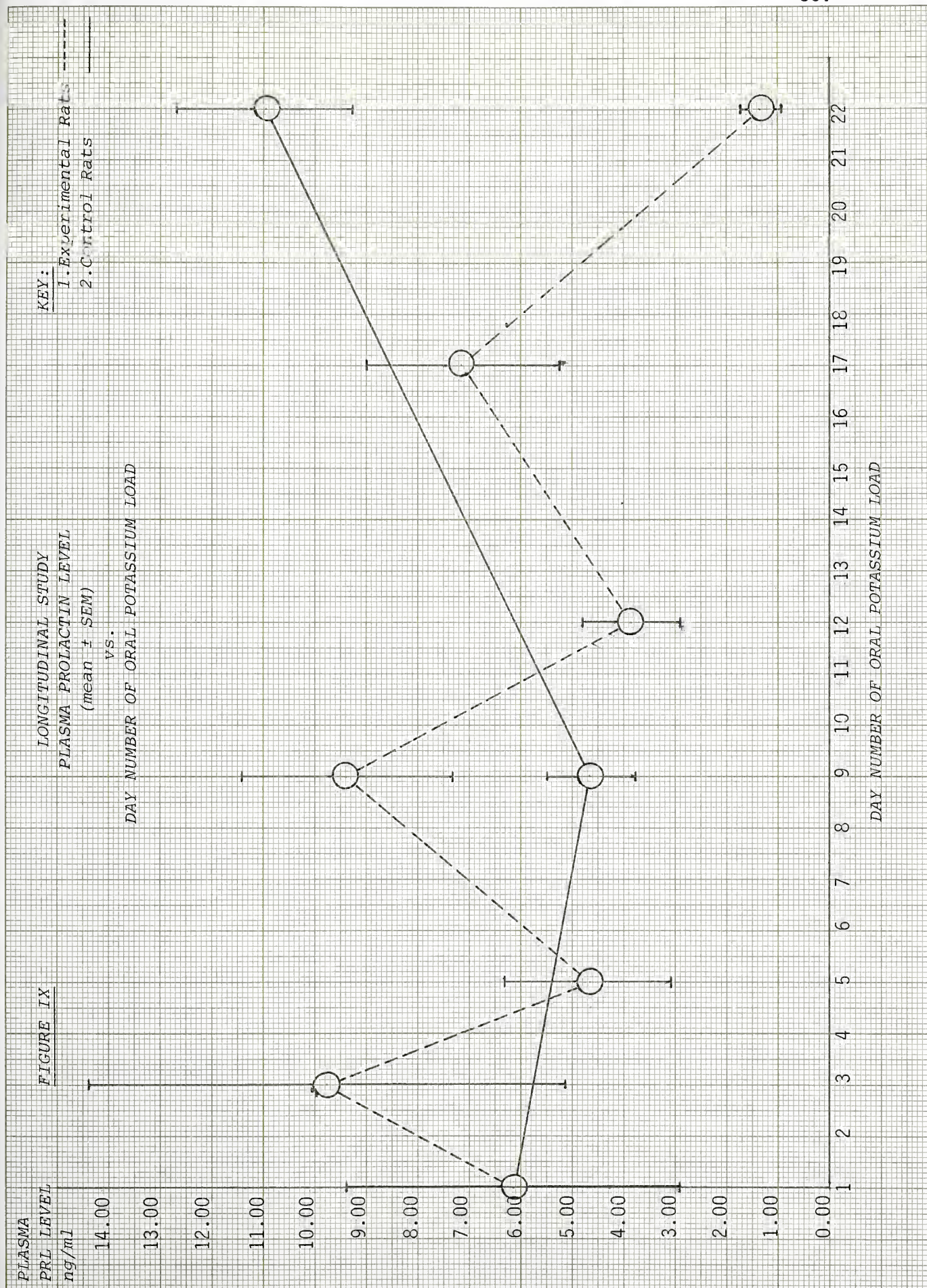
LONGITUDINAL STUDY  
STANDARD CURVE  
RAT PROLACTIN  
E31, 32, 34, 36  
C11-C13  
POOLED PLASMA

FIGURE VIII



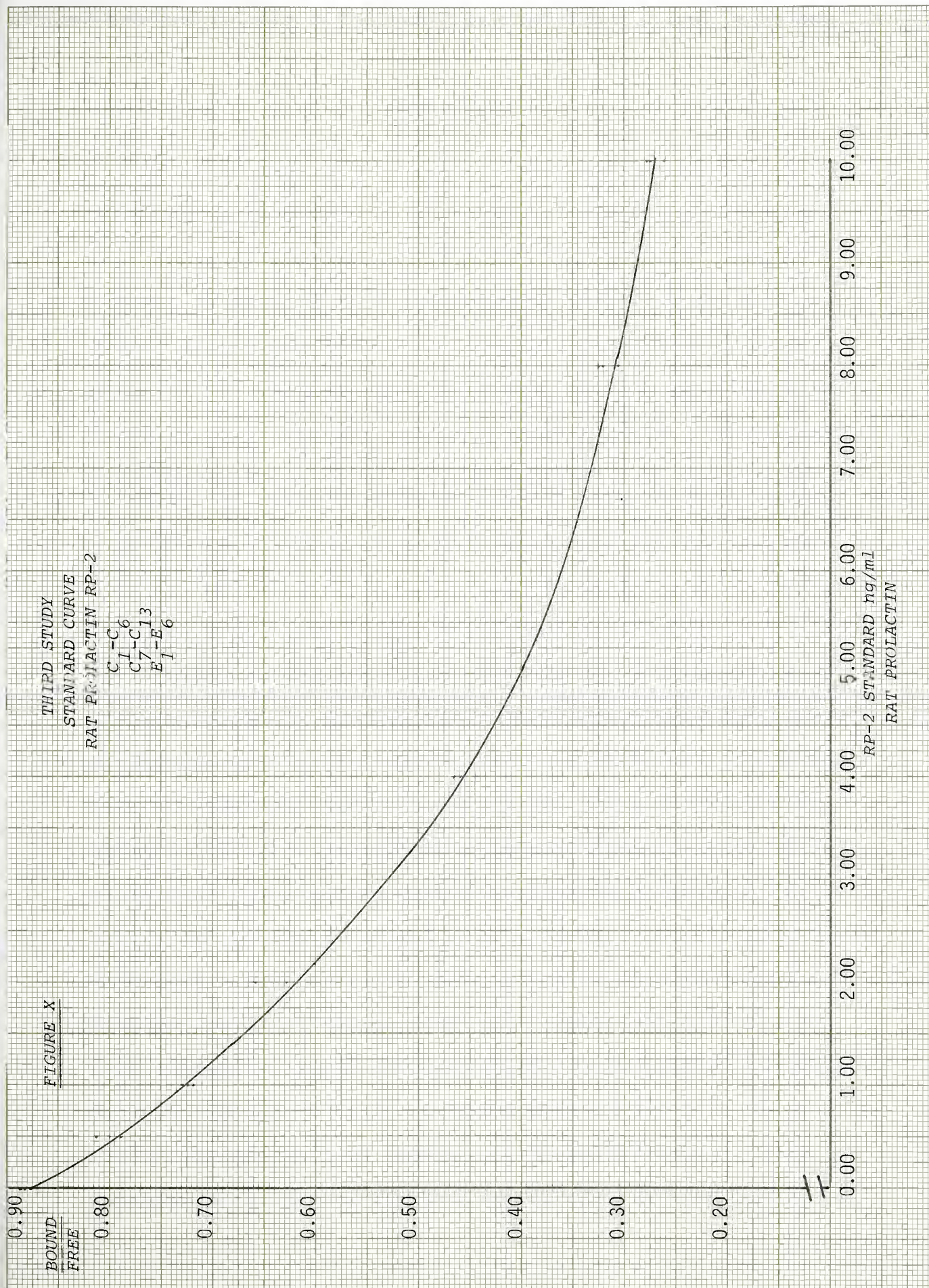
















## DISCUSSION

Increased synthesis and release of pituitary prolactin appears to coincide with the transfer of euryhaline teleost fish from hypertonic to hypotonic environments.<sup>10,11,12</sup> Moreover, only hypophysectomized fish given prolactin replacement or intact fish are able to maintain hydromineral balance upon transfer between these environments.<sup>3,6,7,16</sup> Prolactin increases sodium retention and increases excretion of water permitting adaptation to hypotonicity. The osmoregulatory functions of prolactin in higher vertebrates and factors controlling circulating prolactin levels during osmoregulatory stress are less well understood in mammals.

### I. CONTROL OF CIRCULATING PROLACTIN CONCENTRATIONS IN THE RAT

Circulating prolactin is known to fluctuate in response to a wide variety of stimuli in the rat.<sup>110,111</sup> Neurotransmitters, particularly catecholamines, play a key role in modulating the release of pituitary prolactin. Dopamine appears to inhibit the release of prolactin directly and through stimulation of prolactin inhibitory factor (PIF) in the hypothalamic region. Serotonin appears to stimulate prolactin release, perhaps through modulation of prolactin releasing factor (PRF). Thyrotropin releasing hormone (TRH), located in the hypothalamus, stimulates prolactin release. Prolactin release also appears to be under feedback control of prolactin itself. Meites, et al<sup>111</sup> suggest that this feedback mechanism involves prolactin stimulation of hypothalamic dopamine activity leading to increased PIF release. Much investigation has been performed on the influence of psychotropic



drugs and other chemical agents on these mechanisms of control over prolactin release. Unfortunately, little investigation has been reported on the relation between osmoregulatory stress and these neurogenic mechanisms.

In an early study, Buckman, et al<sup>112</sup> demonstrated a direct relationship between serum prolactin and serum osmolality in humans. In both oral and intravenous administration of hypotonic saline, serum prolactin levels fell to a minimum of 35% baseline prolactin. Conversely, infusion of 5% NaCl 3 ml/kg BW resulting in greater than 10 mosm/kg rise in each subject induced a 158 to 1073% rise in serum prolactin above the baseline values. Correlation by linear regression between serum osmolality and serum prolactin was highly significant (correlation coefficient 0.732,  $P < 0.001$ ). Changes in serum prolactin levels appeared independent of volume expansion alone as determined by isotonic saline infusion. Relkin, et al<sup>113</sup> demonstrated direct relationship between serum osmolality and plasma prolactin in the male rat. Rats infused intravenously with 10 mg 0.45%, 0.9%, 3% NaCl/kg BW/h over 30 minutes were noted to have plasma prolactin levels of  $5 \pm 0.9$ ,  $29 \pm 2.6$ ,  $146 \pm 5.8$  ng/ml plasma (mean  $\pm$  SEM) respectively with corresponding serum osmolalities of  $297 \pm 1.4$ ,  $304 \pm 1.1$ ,  $317 \pm 1.3$  mosm/kg (mean  $\pm$  SEM). Both plasma prolactin and serum osmolality in hypo and hypertonically infused animals were significantly different from the control group ( $P < 0.01$ ). In this investigation (Table I, Table IX and Figure IX), plasma prolactin levels were noted to be 78-79% lower in experimental rats compared to control after approximately three weeks of oral potassium load. On day seven of potassium adapta-



tion in the longitudinal study, there is a rise in plasma prolactin compared to control although not significant at the one percent level (Figure IX, Table IX). Mean serum osmolality in the third study was significantly higher ( $P < 0.01$ ) after five days of potassium load in the experimental group ( $292 \pm 1.80$ ) compared to control ( $284 \pm 0.91$  mosm/kg, mean  $\pm$  SEM). It is possible that a transient rise in circulating prolactin in response to a rise in serum osmolality induces an anti-diuretic effect. This could be followed by a subsequent fall in serum osmolality and an equilibration of circulating prolactin at an appropriately low level. Unfortunately, serum osmolality was not measured after more than five days of potassium adaptation. Moreover, Mattheij, et al<sup>115</sup> failed to demonstrate a rise in plasma prolactin in response to 5% NaCl 2.5 ml hypertonic saline infusion. In additional hydration and dehydration studies, Mattheij, et al conclude that osmolality is not important in regulating circulating prolactin levels in the adult rat.<sup>118</sup>

Acute stress stimuli (e.g., cardiac puncture, ether anesthesia) elevate prolactin in the male rat.<sup>101,102</sup> Tache, et al<sup>116</sup> demonstrated a significant decrease in plasma prolactin levels in rats exposed to chronic stress. After 3, 15 and 42 days of daily exposure to cold for six hour periods, the decrease in plasma prolactin levels ranged from 61% to 82% compared to controls ( $P < 0.05$  to 0.001). Similarly, after 6, 15 and 42 days of daily forced exercise for six hour periods, the decrease in plasma prolactin levels ranged from 56 to 68% ( $P < 0.05$  to 0.001). Adrenal weights were higher in the chronic stress groups, suggesting stimulation of adrenocortical activity possibly through in-





creased ACTH secretion. The relation between ACTH and prolactin secretion is unclear. Long term oral potassium load may represent a chronic stress stimulus to the male rat through starvation (experimental rats weighed only one-half the control group weight although daily food intake appeared identical in the longitudinal study) or perhaps through development of a hypermetabolic state.

Sodium deprivation and potassium loading increase serum aldosterone levels in rats.<sup>86</sup> Relkin and co-workers<sup>114</sup> demonstrated a rise in plasma prolactin during sodium deprivation in contrast to later work by Mattheij who showed no change in plasma prolactin.<sup>115</sup>

However, in both sodium deprivation studies, plasma aldosterone levels or aldosterone secretory rates were markedly elevated in experimental animals. Relkin suggested a possible relationship mediated by serum osmolarity between increased plasma prolactin levels and aldosterone secretory rates in long term sodium deprivation. Although serum osmolarity was not measured in this study, a rise in hematocrit was interpreted as an indication of dehydration and increased serum osmolality. Meites, et al<sup>57</sup> reported an increase in plasma prolactin in rats deprived of water for 48 hours in addition to a rise in specific binding of prolactin to whole adrenal microsomal preparations and a fall in binding to kidney microsomal preparations. The relationship between aldosterone secretion, serum osmolality and plasma prolactin is unclear due to conflicting results reported by different investigators. Depressed plasma prolactin levels and increased adrenal weight in response to chronic stress appear to conflict with the reported direct relationship between plasma prolactin and aldosterone levels.



It is quite possible that these hormonal fluctuations are unrelated except for their independent responses to common stimuli.



## II. PHYSIOLOGIC EFFECTS OF PROLACTIN ON THE MAMMALIAN KIDNEY

As discussed in the introduction, prolactin has been shown to decrease urinary sodium and potassium excretion as well as reduce urine formation in the mammalian kidney.<sup>60,61,62,65</sup> Lockett and co-workers<sup>61</sup> demonstrated an initial natriuresis and diuresis followed by a reduction in sodium and potassium excretion and an antidiuresis in response to a 30 µg ovine lactogenic hormone infusion in the headless cat model. Lucci, et al<sup>60</sup> showed prolactin infusion (approximating the female rat proestrus circulating prolactin level) inhibited diuresis and natriuresis during volume expansion with intravenous normal saline infusion in the rat. Fractional proximal sodium reabsorption is reduced during a saline expansion.<sup>60</sup> This evidence taken together suggested that prolactin exerts its renal effects on the proximal tubule.

Lockett, et al also demonstrated potentiation of aldosterone mediated antinatriuresis and antidiuresis in the headless cat system. Infusion of 0.25 µg aldosterone increased urine flow and sodium excretion. Subsequent infusion of 12.5 µg of bovine lactogenic hormone reversed this initial effect. Prolactin is therefore thought to modulate aldosterone effects in the distal nephron.<sup>60,118</sup>

Prolactin potentiates the antidiuretic effects of vasopressin in certain experimental conditions. Cortisol 50 mg IM injections given three times daily reverses the water retaining effect of vasopressin 15 mg IV given once a day to a diuretic effect. This reversal in sheep is corrected by administration of ovine prolactin 5 mg IM.<sup>119</sup> Similarly, in lithium treated rats, vasopressin 2.5 µg fails to produce an antidiuresis. Although prolactin 250 µg alone also fails to





produce antidiuresis, when vasopressin and prolactin are given in the same doses respectively, a marked reduction in urine formation occurs.<sup>120</sup>

Wallin, et al<sup>59</sup> demonstrated a marked decrease in free water clearance in the rat in response to infusion of ovine prolactin at 200 nmol/kg BW/h. Rats receiving infusions of aqueous pitressin at 75 mμ/kg BW/h demonstrated no further decrease in free water clearance in response to ovine prolactin infusion. In addition, infusion of ovine prolactin, rat prolactin and vasopressin to produce maximum urinary osmolality resulted in significant increases in urinary cAMP. Both ovine and rat prolactin preparations were determined free of vasopressin contamination by column chromatography.<sup>59</sup> This suggested direct antidiuretic effect of prolactin, most likely at the collecting duct region of the rat distal nephron. Autoradiographic studies localized prolactin binding to the ascending thick limb of the distal tubule and the collecting duct.<sup>58</sup> Whether prolactin is binding at the same receptor as vasopressin is uncertain.

The interactions between adrenal steroids and prolactin are not readily understood in terms of the aforementioned physiologic effects of prolactin on mammalian renal function. Glucocorticoids reduce specific prolactin binding to rat kidney membrane preparations in a dose related manner.<sup>122</sup> However, in adrenalectomized rats, corticosterone 133 μg/100 g BW given for eight days restores prolactin induced decrease in sodium and potassium excretion and increase in free water clearance observed in intact rats.<sup>123</sup> (The increase in free water clearance in prolactin treated rats conflicts with results in previous studies.) Scatchard analysis suggests the reduction in specific prolactin binding



is due to a change in binding capacity rather than changes in receptor affinity. Aldosterone did not effect prolactin binding.

Although prolactin appears to modulate Na-K-ATPase function in mammalian tissues such as guinea pig myometrium,<sup>68</sup> rabbit mammary gland,<sup>69,70,71</sup> and rat jejunum,<sup>121</sup> there is no direct evidence that prolactin exerts an influence on mammalian renal function through Na-K-ATPase activity.



### III. EXPERIMENTAL DESIGN FOR MEASURING RAT PROLACTIN

#### A. Effects of Stress

Acute stress stimuli elevate and chronic stress stimuli depress plasma prolactin in the rat. Although care was taken to minimize acute stress stimuli in this investigation, it is difficult to account for all possible stimuli leading to increased plasma levels. Similarly, the effect of chronic stress is particularly important in potassium adaptation. The depressed mean plasma prolactin levels in the pilot and the longitudinal studies may be directly related to the chronic stress of potassium or to secondary effects of potassium adaptation (e.g., failure to gain weight compared to controls).

#### B. Variability in a Biologic System

The mean plasma prolactin levels of experimental groups in the longitudinal study (Table IX, Figure IX) illustrate the variability of circulating prolactin levels. The difference between E<sub>19</sub>-E<sub>24</sub> and E<sub>13</sub>-E<sub>18</sub> is statistically significant at the 5 percent level. This intergroup variation brings the validity of assuming straight lines between the points and hence linear regression analysis into question. Increasing the number of rats in each group may have reduced this variability although it is possible that stimuli to prolactin secretion remain unaccounted for.





### CONCLUSION

The control role of prolactin in modulating osmoregulatory functions in lower vertebrates has been reviewed. Many of the effects of prolactin on major osmoregulatory tissues in the euryhaline teleost, particularly the kidney, parallels the morphologic and physiologic changes observed in the rat kidney and colon in response to a variety of stimuli. The osmoregulatory functions of prolactin in mammalian tissues is ill-defined although it appears to have direct antidiuretic, antikuliuretic and antinatriuretic effects. It also plays a permissive role for other hormones to exert their major effects including aldosterone and vasopressin. The actual mechanisms involved in these hormonal interactions have yet to be solved. However, the parallel between prolactin's effects in the euryhaline fish and the changes observed in the rat ion transporting epithelium in response to a variety of seemingly unrelated stimuli raises the question of yet another permissive role of prolactin. Specifically, is prolactin involved in mediating the osmoregulatory effects of chronic potassium load independent of mineralocorticoid, sodium depletion, surgical ablation, and administration of mineralocorticoid or glucocorticoid on rat kidney or colon?

This study failed to demonstrate elevated circulating prolactin levels in chronic potassium loaded rats. On the contrary, a fall in plasma prolactin was demonstrated after approximately three weeks of potassium adaptation. Although previous studies have correlated serum osmolalities with circulating prolactin levels, it is doubtful that



serum osmolalities were significantly different in experimental rats after three weeks of potassium adaptation since tap water was offered ad lib. Previous studies have failed to demonstrate elevated serum potassium concentrations required to change serum osmolality.<sup>79</sup> It is difficult to describe the mechanism of a feedback control system active over a prolonged time scale as outlined in the discussion section (page 87).

In summary, the depressed plasma prolactin levels observed after three weeks of oral potassium load most likely represents a response to chronic stress. This investigation fails to establish a role for prolactin in mediating morphologic and physiologic response of rat ion transporting epithelium to various stimuli including chronic oral potassium load. Administration of a super-physiologic prolactin dose and studying changes in rat kidneys or colon may clarify the osmoregulatory role of prolactin in the rat system.



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